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THE USE OF A PHENANTHRIDINIUM COMPOUND (No. 897) IN THE TREATMENT OF ZEBU CATTLE INFECTED WITH *TRYPANOSOMA CONGOLENSE*

By

H. E. Hornby, S. A. Evans and J. K. H. Wilde,

Veterinary Research Laboratory, Mpwapwa, Tanganyika Territory

INTRODUCTION

Following the appearance of a short paper by Browning, Browning and Robb (1940), indicating the trypanocidal action of a substance 7 amino-9 (*p*-aminophenyl)-10 methyl phenanthridinium chloride, (897), we received a supply of the drug for trial in Africa on bovine trypanosomiasis. Substance 897 is a red powder of fine crystalline consistency. It is soluble in hot water to the extent of 1 per cent., forming a solution that is clear, stable, and of a deep orange colour. This solution cannot be achieved easily with the use of cold water alone, and it represents, therefore, the upper limit of concentration for practical use. The pH of this solution appears to be just above 7·0. Although Browning recommended that a concentration not higher than 1 : 1,000 should be used for subcutaneous or intramuscular injection, there are practical reasons why, for extensive veterinary field use, the volume of an injection should be small: the injection of a large dose in fractions and dispersion of the fluid by thorough massaging is difficult in such conditions. The injection of a very large volume is not altogether impracticable, but before resorting to it we wished to know whether the ill-effects from the use of a more concentrated solution outweigh the great advantage of easy administration. Our observations have therefore been confined to solutions of from ½ to 1 per cent., as these concentrations allowed injections that did not exceed 80 c.c.—*i.e.*, a couple of 40 c.c. syringe-fulls. Our first observations were directed towards obtaining some idea of what was likely to be an effective yet safe quantity to administer, and we chose a dose of 10 c.c. of 1 per cent. aqueous solution per cwt. (approximately 2 mg. per kilo.). During the first series of experiments this was considered the "normal" dose.

Before describing the experiments we must make clear what we mean by "cure." Treated animals have their blood examined microscopically every week, and an animal is not discharged as cured until it has undergone such examinations, yielding negative results, for a period of six months since the last appearance of trypanosomes in its blood. In addition it must show good condition and a reasonably high blood haemoglobin value, having due regard to such factors as seasonable changes in environment. Such a beast is clinically

cured, but is not necessarily sterilised; it may be merely strongly premunised and unlikely to relapse.

PRELIMINARY EXPERIMENTS: SERIES A

Case histories in these early experiments are not given in detail, but the results obtained are stated and discussed. In all cases the trypanosome concerned is *T. congolense*, and with one exception the animals are zebus.

(1) *The effect of a subcutaneous injection of a single "normal" dose.*—Three oxen, which had previously relapsed after a full course of Tartar Emetic treatment, were given 897 subcutaneously. Two of these animals were cured without further treatment, although they showed very severe subcutaneous reactions. The third animal relapsed within a fortnight, but showed a negligible local reaction.

(2) *The effect of a "double" dose given subcutaneously* (17 c.c. of a 1 per cent. solution per cwt.).—One animal only was tested in this way, and although the trypanosomes disappeared within 48 hours, they reappeared within three weeks. A large swelling arose at the site of the injection and persisted until the animal was slaughtered two months later. At *post-mortem* the skin at the site of the injection was very thick and blistered, and firmly adherent to the underlying tissues, which were partially bruised, partially in a state of gelatinous necrosis, but chiefly fibrosed.

(3) *The effect of a single "normal" dose given intramuscularly.*—Twenty bovines, naturally infected with *T. congolense*, many of which had been treated unsuccessfully with Tartar Emetic, were given a single intramuscular injection of 897 at the rate of 10 c.c. of a 1 per cent. solution per cwt., the injection being made into the thick gluteal mass of one quarter. None had received the drug in any form before. Fourteen were cured; five relapsed; and one was doubtfully cured in that it did not regain good condition during the six months of freedom from trypanosomes. A slight local reaction at the site of intramuscular injection is inevitable, but it disappears after varying periods. When the 14 cured animals were discharged six to seven months after treatment, in only three was there more than a suggestion that there had ever been a lesion. In no case did marked lameness result from the swelling or the injection give rise to systemic disturbance.

(4) *The effect of a single "normal" dose given intravenously to an ox relapsed after previous treatment with* 897.—We had previously found that a small dose of 4 c.c. of a 1 per cent. solution per cwt. given intravenously was innocuous, whereas a massive dose of 20 c.c. per cwt. given to a cow was immediately fatal. We tried, therefore, the "normal" dose. The ox treated had relapsed to previous treatment with 897, and it relapsed again, after this intravenous injection, within a fortnight. No further test was made of treatment by a single intravenous dose. It was considered that this was unlikely

to give better results than a single intramuscular injection, which, for everyday practice, is preferable. Therefore, when further intramuscular injections become impracticable owing to local reactions, it is unlikely that anything less than a *course* of intravenous injections will effect a cure.

(5) *The effect of five weekly injections of a "normal" dose, given intravenously.*—Three animals which had relapsed to previous treatment with 897, were given a course of five weekly injections at the rate of 10 c.c. of 1 per cent. solution per cwt. intravenously. Two of these animals were discharged as cured six months later, but the third, which had relapsed in experiments 1, 3 and 4, relapsed again a fortnight after this course was completed, and was put on to treatment by another drug.

(6) *The effect of two "normal" doses, given intramuscularly, with a week's interval.*—Two bulls were treated in this manner. One had relapsed to previous 897 treatment, and the other was a case of fresh infection. The former relapsed again after five weeks, whereas the other was cured by the two injections. Had both cases been cured we should have gone on with the trial. As it was, the result of this experiment left us inclined to treat each fresh case with a single intramuscular dose.

DISCUSSION OF SERIES A

This series of experiments was really in the nature of a preliminary trial of the drug, and formed a useful guide for our subsequent work. The series involved the testing of 897 under different methods of administration.

Our original "normal" dose, defined as 10 c.c. of 1 per cent. solution per cwt. body weight, was tested in the following ways: (*a*) a single subcutaneous injection; (*b*) a single intramuscular injection; (*c*) a single intravenous injection; (*d*) five intravenous injections at weekly intervals; (*e*) two intramuscular injections with an interval of one week. Also a single injection of almost twice the "normal" dose was tried subcutaneously.

The trypanocidal action of 897 was confirmed, trypanosomes generally disappearing from the blood stream within 24 hours of the administration of the drug. No other definite conclusions were drawn from this series of experiments, but using our observations as a basis, further experimental treatment was planned. It was thought that the solution of 1 per cent. might be unnecessarily concentrated and that it would be advisable, if practicable, to reduce the concentration without unduly increasing the bulk of the injection, by reducing the dose. We were prompted to adopt this course because although intramuscular injections did not cause noticeable lameness, they left a necrotic track in the gluteal muscles, surrounded by an area of discoloration, with a thickening of the subcutaneous fascia. This detracts to a certain extent from the meat value of a

carcase, especially if both quarters have been injected. The dose, therefore, upon which we agreed for further experiment was 15 c.c. of a 0·5 per cent. solution per cwt. body weight, and we shall refer to this in connection with the next series of experiments as the " routine " dose. We found this dose was sufficient to cause disappearance of all trypanosomes from the blood stream of an infected ox within 24 hours of administration, and we planned the following treatment for the next (B) series of cases : (*a*) One " routine " dose to be injected intramuscularly into one quarter. (*b*) If relapse occurred, the dose to be repeated into the other quarter. (*c*) If relapse occurred again, a course of three " normal " doses (*i.e.*, 10 c.c. of 1 per cent. solution per cwt.) to be given intravenously, at weekly intervals.

SERIES B

The subjects in this series were : six zebu cattle naturally infected with *T. congolense*, and 14 zebu oxen and one zebu cow, all inoculated with blood from a naturally occurring case. These animals, which were all inoculated at the same time, were not all treated immediately infection was established. Some were kept for a varying number of weeks after showing trypanosomes in the blood, before treatment was started, to see if the consequent development of antibodies more than compensated for the disadvantages of increased anaemia and weakness.

The course of treatment for each animal is given as briefly as possible in the Appendix. The details are so arranged that the course and the results may be seen at a glance and compared with the animal's weight, the latter acting as a fairly reliable guide to condition. In two or three cases the recommended procedure was modified slightly, but not sufficiently to prevent them being included in the following summary of results :—

Of the six naturally infected cases, four were cured, and two probably cured.

Of the 15 experimentally infected cases, eleven were cured, one was probably cured, and three were definitely not cured.

Of the 18 cured or probably cured animals, eight received a single intramuscular injection, four received two intramuscular injections, three received two intramuscular injections and one intravenous injection, three received two intramuscular injections and three intravenous injections.

The results are sufficiently good to render unnecessary any discussion concerning the advisability or otherwise of allowing the disease to take full hold before commencing treatment, though it may be noted that the three failures were all treated early, five weeks or less after inoculation. More fruitful is a discussion as to whether these failures represent the achievement of drug-fastness by the trypanosomes.

DRUG-FASTNESS

Reference to the case sheets will show that one of the oxen, No. 6742 of Series B, that failed to respond to treatment with 897, continued to show trypanosomes throughout the latter part of the full course of treatment, so before putting it on to treatment with another drug its blood was inoculated into a clean ox, No. 2310. This animal developed a heavy infection of *T. congolense* within 14 days. Eight days later it was given a massive dose of 897 (1 per cent. solution at the rate of 15 c.c. per cwt.) intravenously; yet a week later its blood showed trypanosomes. It was then put on to treatment with Stibophen, and eventually died from drug poisoning.

Similar qualified evidence of drug-fastness is given by the result of inoculating ox No. 3815 from cow No. 8. This was a case of natural infection that recovered after a single intramuscular injection of 897, given on 27.6.41. She was discharged cured six months later. By 2.4.42 she had become reinfected (though the possibility of a relapse could not be excluded), and this time she failed to respond to three injections of 897 at weekly intervals. Blood was drawn on 24.4.42, and inoculated into ox No. 3815. A fortnight after inoculation its blood showed a heavy *T. congolense* infection, and eight days later it was given a massive dose of 897 (1 per cent. solution at the rate of 15 c.c. per cwt.) intravenously. Trypanosomes disappeared for a short time, but had reappeared within a fortnight. Thereafter it was treated and cured by a course of Stibophen.

In a more comprehensive experiment three oxen, Nos. 3489, 6419 and 6426, were inoculated, each with 10 c.c. of citrated blood drawn from ox No. 25 at a time when this animal was heavily positive after relapsing several times to 897 treatment. No treatment was commenced until the trypanosomes were well established in all three beasts, five weeks after inoculation. Treatment was then started. One animal received 897, and the other two Stibophen and M. & B. 744, respectively. The case histories are given in the Appendix. From these history sheets it will be seen that oxen Nos. 6419 and 6426, which were treated with Stibophen and 744 respectively, showed a few trypanosomes in the blood just after treatment, but not subsequently. These drugs, therefore, may be assumed to have been effective in some degree against the trypanosomes. No. 3489, however, which was treated with 897 and received the full course of two intramuscular and three intravenous injections, relapsed 14 days after completion of this treatment and received a further intravenous dose of 897. Trypanosomes were still present, however, when the blood was examined six days later, and persisted until a further intravenous injection was given seven days after that. The following week, trypanosomes were again present, and two more intravenous doses were given at a week's interval. Again, after the lapse of only a week, trypanosomes were

demonstrable in the blood, and they continued to appear at intervals during the next three and a half weeks, when no treatment was given. At the end of this period a large final dose of 897 was given intravenously (60 c.c. of 1 per cent. solution, instead of 50 c.c.) and the animal's blood was examined every few hours. Trypanosomes failed to disappear within four days. Treatment with Antimosan was then started, and the parasites disappeared from the blood stream less than two and a half hours after the first subcutaneous injection.

Conclusions regarding fastness.—From these observations we conclude that in some cases of *T. congolense* infection, treatment with 897 may induce resistance to the drug in the parasites, but such fastness is specific to 897, and does not prevent subsequent successful treatment with other drugs. To reduce the danger of producing fastness, unnecessarily small doses should be avoided, and for this reason the " routine " dose recommended, namely, 15 c.c. of ½ per cent. solution per cwt., should also be considered minimal.

GENERAL CONCLUSIONS

From the experiments described above, and all our experience with the drug 7 amino-9 (*p*-aminophenyl)-10 methyl phenanthridinium chloride, extending over 20 months, we are of the opinion that this drug has a place among the more valuable of our trypanocidal agents. In most cases of *T. congolense* infection of zebus it is rapidly effective, and its value is enhanced by the fact that it can be given intramuscularly without causing serious lesions or appreciable lameness.

We recommend that intramuscular injections be at the rate of 15 c.c. of ½ per cent. aqueous solution per cwt. body weight, and intravenous injections at the rate of 10 c.c. of 1 per cent. solution per cwt. A suggested routine treatment is as follows. One intramuscular injection into one quarter. If relapse occurs, the dose to be repeated, using the other quarter. If relapse occurs again, a course of three intravenous injections at weekly intervals. If this full course fails to effect cure, another trypanocidal drug should be tried.

ACKNOWLEDGMENT

We are indebted for supplies of the drug to the Chemical Research Laboratory of the Department of Scientific and Industrial Research, Teddington, and the Chemotherapeutic Committee of the Medical Research Council.

REFERENCE

Browning, C. H., Browning, P., and Robb, J. V. M. (1940). *J. Path. Bact.*, **50**, 371.

APPENDIX

Date	*Trypanosomes*	*Treatment*	*Weight* (*cwt.*	*qr.*	*lb.*)
(1) *Ox No. 4.* Natural infection.					
7. 2.42	*+	75 c.c. ½ per cent. 897 i.m.	5	0	0
21. 5.42	+	75 c.c. ½ per cent. 897 i.m.	5	0	0
26.11.42		Discharged cured.			
* + = trypanosomes present.					
(2) *Cow No. 7.* Natural infection.					
4. 2.42	+	65 c.c. ½ per cent. 897 i.m.	4	0	14
19. 3.42	+		4	2	0
23. 3.42	+	65 c.c. ½ per cent. 897 i.m.			
15.10.42		Discharged cured.	4	3	19
(3) *Ox No. 8.* Natural infection.					
7. 2.42	+	60 c.c. ½ per cent. 897 i.m.	3	3	7
15.10.42		Discharged cured.	5	0	13
(4) *Ox No. 11.* Natural infection.					
23.12.41	+	65 c.c. ½ per cent. 897 i.m.	4	0	21
15.10.42		Discharged cured.	5	0	0
(5) *Cow No. 20.* Natural infection. History of previous infection cured by course of 897.					
10. 4.42	+	75 c.c. ½ per cent. 897 i.m.			
9. 5.42	+	75 c.c. ½ per cent. 897 i.m.	5	0	27
22. 5.42	+	40 c.c. 1 per cent. 897 i.v.	4	3	21
29. 5.42		40 c.c. 1 per cent. 897 i.v.	5	1	4
5. 6.42		40 c.c. 1 per cent. 897 i.v.	5	1	12
16. 7.42	+				
Subsequent Note: 26.11.42. Has not shown trypanosomes again.					
(6) *Ox No. 6023.* Natural infection.					
23.12.41	+	75 c.c. ½ per cent. 897 i.m.	4	3	15
13. 3.42	+	75 c.c. ½ per cent. 897 i.m.			
3. 7.42	+	60 c.c. 1 per cent. 897 i.v.	6	1	24
10. 7.42		60 c.c. 1 per cent. 897 i.v.	6	2	3
17. 7.42		60 c.c. 1 per cent. 897 i.v.	6	2	4
Subsequent Note: 26.11.42. Has been free from trypanosomes since last treatment.					
(7) *Ox No. 6740.* Experimental infection.					
4. 2.42		Inoculated from Ox. No. 8.			
19. 2.42	+		3	1	26
21. 2.42		50 c.c. ½ per cent. 897 i.m.			
15.10.42		Discharge cured.	4	0	13
(8) *Ox No. 6751.* Experimental infection.					
4. 2.42		Inoculated from Ox No. 8.			
19. 2.42	+				
26. 2.42	+		3	0	21
6. 3.42	+	50 c.c. ½ per cent. 897 i.m.			
15.10.42		Discharged cured.	4	0	22

Date	Trypanosomes	Treatment	Weight (cwt.	qr.	lb.)
(9) *Ox No. 6752.* Experimental infection.					
4. 2.42		Inoculated from Ox No. 8.			
12. 2.42	+				
19. 2.42	+				
26. 2.42	+		3	0	17
6. 3.42	+	52 c.c. ½ per cent. 897 i.m.			
16. 4.42	+	55 c.c. ½ per cent. 897 i.v.			
1. 5.42	+	60 c.c. ½ per cent. 897 i.m.	3	1	21
26.11.42		Discharged cured.			
Note: On 16.4.42 the 897 was given intravenously by mistake.					
(10) *Ox No. 6753.* Experimental infection.					
4. 2.42		Inoculated from Ox No. 8.			
12. 2.42	+				
19. 2.42	+				
26. 2.42	+				
6. 3.42	+	52 c.c. ½ per cent. 897 i.m.	3	2	0
3. 7.42	+	60 c.c. ½ per cent. 897 i.m.	4	0	21
10. 7.42		40 c.c. 1 per cent. 897 i.v.	4	0	0
17. 7.42		40 c.c. 1 per cent. 897 i.v.			
24. 7.42	+	60 c.c. 1 per cent. 897 i.v.			
Subsequent Note: 26.11.42. This animal is now in good condition and probably cured.					
(11) *Ox No. 6754.* Experimental infection.					
4. 2.42		Inoculated from Ox No. 8.			
19. 2.42	+				
26. 2.42	+		2	2	0
6. 3.42	+	36 c.c. ½ per cent. 897 i.m.			
3. 4.42	+	40 c.c. ½ per cent. 897 i.m.			
9. 5.42	+	25 c.c. 1 per cent. 897 i.v.	3	0	0
15. 5.42		25 c.c. 1 per cent. 897 i.v.	2	3	8
22. 5.42		25 c.c. 1 per cent. 897 i.v.			
2. 7.42	+		2	3	25
9. 7.42	+		3	0	0
16. 7.42	+	Commenced treatment with M. & B. 744.			
(12) *Ox No. 6755.* Experimental infection.					
4. 2.42		Inoculated from Ox No. 8.			
26. 2.42	+		2	3	11
6. 3.42	+	45 c.c. ½ per cent. 897 i.m.			
15.10.42		Discharged cured.			
(13) *Ox No. 6756.* Experimental infection.					
4. 2.42		Inoculated from Ox No. 8.			
19. 2.42	+		3	0	0
26. 2.42	+				
5. 3.42	+				
12. 3.42	+				
19. 3.42	+		3	2	10
24. 3.42	+	60 c.c. ½ per cent. 897 i.m.			
15.10.42		Discharged cured.	4	0	19
(14) *Ox No. 6757.* Experimental infection.					
4. 2.42		Inoculated from Ox No. 8.			
19. 2.42	+				
26. 2.42	+		2	3	12
12. 3.42	+				
19. 3.42	+		3	0	0
24. 3.42	+	45 c.c. ½ per cent. 897 i.m.			
16. 4.42	+	50 c.c. ½ per cent. 897 i.m.	3	0	3
23.10.42		Discharged cured.	3	3	1

Date	Trypanosomes	Treatment	Weight (cwt.	qr.	lb.)
(15) *Ox No. 6758.* Experimental infection.					
4. 2.42		Inoculated from Ox No. 8.			
12. 2.42	+				
19. 2.42	+				
26. 2.42	+		3	1	21
5. 3.42	+				
12. 3.42	N.T.S.				
19. 3.42	+				
23. 3.42	+	50 c.c. ½ per cent. 897 i.m.	3	2	0
24. 4.42	+	60 c.c. ½ per cent. 897 i.m.			
30. 4.42	+	40 c.c. 1 per cent. 897 i.v.	3	2	0
9. 5.42		40 c.c. 1 per cent. 897 i.v.	3	2	15
15. 5.42		40 c.c. 1 per cent. 897 i.v.	3	2	10
26.11.42		Discharged cured.			
N.T.S. = No trypanosomes seen.					
(16) *Ox No. 6759.* Experimental infection.					
4. 2.42		Inoculated from Ox No. 8.			
19. 2.42	+				
26. 2.42	+		3	1	14
5. 3.42	+				
12. 3.42	+				
19. 3.42	+		3	1	15
23. 3.42	+	52 c.c. ½ per cent. 897 i.m.			
24. 4.42	+	60 c.c. ½ per cent. 897 i.m.			
23.10.42		Discharged cured.	3	3	13
(17) *Ox No. 6760.* Experimental infection.					
4. 2.42		Inoculated from Ox No. 8.			
12. 2.42	+				
26. 2.42	+				
5. 3.42	+		3	0	3
12. 3.42	+				
19. 3.42	+		3	0	0
23. 3.42	+	50 c.c. ½ per cent. 897 i.m.			
15. 5.42	+	60 c.c. ½ per cent. 897 i.m.			
22. 5.42	+	25 c.c. 1 per cent. 897 i.v.	3	0	1
29. 5.42		Not injected, owing to phlebitis.			
26.11.42		Discharged cured.			
(18) *Ox No. 6762.* Experimental infection.					
4. 2.42		Inoculated from Ox No. 8.			
26. 2.42	+		2	3	16
5. 3.42	+				
12. 3.42	+				
19. 3.42	+		2	3	14
23. 3.42	+	45 c.c. ½ per cent. 897 i.m.			
15.10.42		Discharged cured.	3	2	16
(19) *Ox No. 6764.* Experimental infection.					
4. 2.42		Inoculated from Ox. No. 8.			
26. 2.42	+		2	3	0
5. 3.42	+				
12. 3.42	+				
19. 3.42	+		2	3	18
23. 3.42	+	60 c.c. ½ per cent. 897 i.m.			
15.10.42		Discharged cured.	3	2	15

Date	*Trypanosomes*	*Treatment*	*Weight* (*cwt.*	*qr.*	*lb.*)
(20) *Cow No. 4.* Experimental infection. History of previous infection and incomplete treatment with 897.					
4. 2.42		Inoculated (in error) from Ox No. 8			
12. 2.42	+				
21. 2.42	+	60 c.c. ½ per cent. 897 i.m.	3	3	21
2. 4.42	+	60 c.c. ½ per cent. 897 i.m.			
1. 5.42	+	35 c.c. 1 per cent. 897 i.v.	4	0	17
9. 5.42		35 c.c. 1 per cent. 897 i.v.			
15. 5.42		35 c.c. 1 per cent. 897 i.v.	4	1	10
4. 6.42	+				
10. 7.42	+	Commenced treatment with Stibophen	3	3	18
(21) *Ox No. 6742.* Experimental infection.					
4. 2.42		Inoculated from Ox No. 8.			
12. 2.42	+				
21. 2.42	+	60 c.c. ½ per cent. 897 i.m.	3	3	0
23. 3.42	+	60 c.c. ½ per cent. 897 i.m.			
3. 4.42	+	60 c.c. ½ per cent. 897 i.v.			
10. 4.42	+	60 c.c. ½ per cent. 897 i.v.			
17. 4.42	+	60 c.c. 1 per cent. 897 i.v.			
23. 4.42	+	Commenced treatment with M. & B. 744	3	3	22
(22) *Ox No. 3815.* Experimental infection. Inoculated from Cow No. 8, whose *T. congolense* were believed to be 897-fast.					
24. 4.42		Inoculated from Cow No. 8	7	0	0
7. 5.42	+		6	3	15
14. 5.42	+	100 c.c. 1 per cent. 897 i.v.	6	3	23
28. 5.42	+	Commenced treatment with Stibophen	7	0	10
24.12.42		Discharged cured.	7	0	0
(23) *Ox No. 25.* Natural infection. Relapsed after full course of tartar emetic.					
6. 6.41	+	50 c.c. 1 per cent. 897 i.m.	5	0	21
24. 7.41	+	53 c.c. 1 per cent. 897 i.m.			
21.11.41	+	80 c.c. ½ per cent. 897 i.m.	5	0	15
17. 4.42	+	80 c.c. ½ per cent. 897 i.v.	5	3	19
15. 5.42	+	80 c.c. ½ per cent. 897 i.v.			
26. 6.42	+	60 c.c. 1 per cent. 897 i.v.	6	0	15
3. 7.42	+	60 c.c. 1 per cent. 897 i.v.	6	0	15
10. 7.42	+	60 c.c. 1 per cent. 897 i.v.	6	0	0
16. 7.42	+				
23. 7.42	+		5	3	26
30. 7.42	+		6	0	7
6. 8.42	+	Blood drawn for inoculation of Oxen No. 3489, 6419 and 6426.			
7. 8.42	+	Commenced treatment with M. & B. 744.			

Note: It was considered that the trypanosomes had become 897-fast.

Date	Trypanosomes	Treatment	Weight (cwt.	qr.	lb.)
(24) *Ox No. 3489.* Experimental infection with presumed 897-fast *T. congolense.*					
6. 8.42		Inoculated with blood from Ox No. 25	5	3	7
3. 9.42	+		6	0	14
11. 9.42	+	60 c.c. 1 per cent. 897 i.m.	5	3	18
18. 9.42	+	60 c.c. 1 per cent. 897 i.m.			
25. 9.42	+	60 c.c. 1 per cent. 897 i.v.	5	3	5
2.10.42	+	60 c.c. 1 per cent. 897 i.v.	5	2	9
9.10.42		60 c.c. 1 per cent. 897 i.v.	5	2	23
23.10.42	+	60 c.c. 1 per cent. 897 i.v.	5	2	3
29.10.42	+				
6.11.42	+	55 c.c. 1 per cent. 897 i.v.			
12.11.42	+	55 c.c. 1 per cent. 897 i.v.	5	1	8
20.11.42		55 c.c. 1 per cent. 897 i.v.	5	0	9
3.12.42	+		4	2	14
24.12.42	+		4	3	4
28.12.42	+	60 c.c. 1 per cent. 897 i.v.			
1. 1.43	+	Commenced treatment with Antimosan.			
(25) *Ox No. 6419.* Experimental infection. Inoculated from Ox No. 25, whose *T. congolense* were presumed to be 897-fast. Compare with Ox No. 3489, similarly inoculated.					
6. 8.42		Inoculated from Ox No. 25	6	0	21
27. 8.42	+		6	0	14
3. 9.42	+		6	0	4
11. 9.42	+	60 c.c. Stibophen s.c.	5	3	26
18. 9.42		60 c.c. Stibophen s.c.	5	3	16
25. 9.42		60 c.c. Stibophen s.c.	5	3	25
29.10.42			6	0	1
5.11.42	+		6	0	20
12.11.42			6	0	7
19.11.42			5	3	18
3.12.42			6	0	0
Subsequent Note: 1.1.43. No trypanosomes seen since 5.11.42.					
(26) *Ox No. 6426.* Experimental infection. Inoculated from Ox No. 25, whose *T. congolense* were presumed to be 897-fast. Compare with Ox No. 3489, similarly inoculated.					
6. 8.42		Inoculated from Ox No. 25	5	2	17
20. 8.42	+		5	2	21
3. 9.42	+		5	2	18
11. 9.42	+	45 c.c. 2 per cent. 744 s.c.	5	2	0
18. 9.42		45 c.c. 2 per cent. 744 s.c.	5	2	1
25. 9.42		45 c.c. 2 per cent. 744 s.c.	5	2	0
5.11.42	+		5	1	20
12.11.42	N.T.S.		5	1	7
19.11.42	+		4	3	21
26.11.42	+		4	3	3
3.12.42	N.T.S.		4	1	21
Subsequent Note: 1.1.43. No trypanosomes seen (N.T.S.) since 26.11.42.					

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A STUDY OF THE ANTIBODY RESPONSE OF CATTLE TO *TRICHOMONAS FOETUS*

By

W. R. Kerr and M. Robertson

From the Ministry of Agriculture of Northern Ireland, the Lister Institute, and the Institute of Animal Pathology, Cambridge

INTRODUCTION

In a previous paper (Kerr and Robertson, 1941) it was shown that the sera of many cattle suffering from infection with *Trichomonas foetus* possess an enhanced agglutinin titre for this parasite. The agglutination test* which revealed this change in the serum was described in detail and the results of its application to a considerable number of cattle were given. It was made clear that all normal adult cattle show a distinct agglutinin titre for *Tr. foetus*. The " positive sera " of infected animals were consequently understood to be those which should have a titre raised above this normal level. Certain animals harboured Trichomonas without any rise in the agglutination titre above the normal level. The test could not, therefore, be relied upon as a means of distinguishing the non-infected individuals. The positive results could thus be regarded as of a diagnostic value but the negative results gave no assurance that the animal was not carrying Trichomonas. Further, while from adult animals which could have had no contact with the disease, no true positive sera have been recorded, a certain number of high " normals " have been found. It seemed worth while to investigate how the enhanced titre against the organism was acquired and its relation to the development of disease.

The Development of the Normal Titre

If the sera of newly born calves of normal mothers are examined shortly after birth, and in particular of calves which have not been given the colostrum, it is found that their sera contain no agglutinins (in dilution 1–6) for living *Tr. foetus*. Within the first days in a few cases there may be a slight amount of agglutinin present, which then disappears. If samples are repeatedly examined as time goes on, there is a gradual, or sometimes slightly irregular, advance in the amount of agglutinin till at some period, usually between the fourth and sixth month, the normal adult titre is acquired. It remains, however, a frequent observation that young animals under one year of age may have rather low titres. It is advisable that sera chosen

* Readers are referred to the previous papers (Robertson, 1941 ; Kerr and Robertson, 1941) for details of the test, but certain points which are useful in the reading of the present account are briefly stated in Appendix I.

for normal controls should be from animals at least one year old. The specificity of the normal agglutinins will be investigated, it is hoped, on some future occasion. No information is available as to how this normal titre is acquired.

Nature of the Disease

All the evidence recorded indicates that trichomoniasis is manifested as a disease only of the genital tract in cattle.* Great pains have been taken to make repeated blood cultures on suitable media from infected cattle, both natural cases in the field and artificially infected animals, but the results have always been negative and no confirmation has been obtained of the claims of Wagner and Hees (1937) and Küst (1936) to have found *Tr. foetus* in the blood. *Trypanosoma theileri*, on the other hand, was frequently cultivated from the blood of both normal and trichomonas-infected animals and this was considered to be a sign that the cultural methods were suitable. The medium used, moreover, was one upon which *Tr. foetus* grew well.

In the survey of field cases positive sera with definitely enhanced titres were found among animals showing return of oestrus at six to nine weeks, with a greater or smaller degree of vaginal discharge in which Trichomonas were found by direct examination. The parasites were also found, however, to be present sometimes at least in certain animals whose titres while " above normal " were not raised to the characteristic positive level. Some of these animals were in calf and later aborted. One well observed case (214) in an infected herd had a very slightly raised titre which would not have been significant on the reading alone had not Trichomonas also been seen in the secretion by a competent observer. The cow aborted at four and a half months and the serum taken five days later had a typical positive titre with index of $16\frac{1}{2}/8\frac{1}{2}$, Trichomonas being again seen in the discharge after abortion. Other cases, however, occurred where the presence of Trichomonas coinciding with the " above normal " titre and subsequent abortion, did not culminate in a further rise in titre. Occasional cases were also recorded where the parasites had been seen by a trustworthy observer, but the serum showed no rise in titre above the normal.

The problem therefore presented was (1) how does any animal get a raised titre in its serum from an infection with a protozoon confined to the genital tract ? and (2) why does not every infection with *Tr. foetus* produce a positive blood titre ? It was decided to approach the matter experimentally. This was done from two aspects : (1) the artificial infection of heifers and cows, and (2) the uterine instillation of a sterile extract of Trichomonas.

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* The clinical features in both the bull and the cow are described in papers by (Kerr, 1942 ; 1943).

I. ARTIFICIAL INFECTION WITH LIVING TRICHOMONAS

The experimental data are incorporated in Table I. In 1939 a strain of *Tr. foetus* was isolated from the uterus of a cow suffering from pyometra on a farm near Belfast. Live Trichomonas from this strain were introduced into the vaginae of five heifers immediately after service by a clean young bull (C9).* The series was called " C " and the results can be summarised as follows : All the animals harboured the parasite for various periods (see Table I) as shown by direct examination of the secretion under the microscope. Nevertheless, four of the animals (C1, C4, C7 and C8) were rendered pregnant by this first service and gave birth to live calves at full term. The fifth animal (C3) was served on 18.7.39, but came into season in three weeks. On being served again (20.9.39) she became pregnant and calved to time. Living Trichomonas were introduced into the sheath of the bull (C9) but did not result in producing a recognisable infection in any of the three heifers subsequently served by him (C2, C6 and C10).

Thus there are here a group of animals infected by the introduction of Trichomonas into the vagina which all showed the parasites for a sufficiently long time for it to be considered an established infection. None of them were acute cases in the clinical sense and none of them became sterile for any length of time. All produced live calves. None of these animals showed a positive reaction in their sera or sufficiently definite changes in the agglutination titre to be significant.

The results are in general agreement with those produced by Andrews and Miller (1938) in America by the artificial infection of heifers. The state brought about resembles the low type of infection that can sometimes be observed in the field and that probably constitutes the latent state of infection from which acute outbreaks gradually arise. Such outbreaks often appear to be unexplained in their origin as these temporary and unimportant symptoms or delays in conception are easily passed over.

The absence of effect on the blood titre in Experiment " C " led in the following year to a modification of the experimental procedure.

INFECTION EXPERIMENTS OF 1941 AND 1942

The methods used come under the following four heads :—

(1) *Parasites introduced into the vagina of virgin heifers after mating.* A4 and A6.

(2) *Parasites introduced into the uterus.* Heifer A5 and cow C10.

C10 had been covered two years previously by bull C9, which had been unsuccessfully exposed to Trichomonas infection. A

* A Dutch strain " Utrecht " was used for one of these animals (C4).

TABLE I

Expt.	*Previous History*	*No. of Animal*	*Type of Infection*	*Trichomonas*	*Result*	*Serological Findings Aggl. Titre*	*Outcome*
C 1939-1940	Virgin heifer	C1	Washed Trichomonas three minutes after service into vagina. Str. Belfast.	Seen at 65 days and for next seven days. Not seen again.	Infection established	No change during course of experiment.	Temporary vaginitis of some severity. Full time healthy calf to first service.
	do.	C3	Washed Trichomonas 24 hours and 72 hours after service into vagina. Str. Belfast.	Seen for 16 days from 7th to 21st day. Not seen again.	Temporary infection for three weeks with return of service.	No change during course of experiment.	Vaginitis with Trichomonas; returns at three weeks, is mated six weeks later without treatment; full-time healthy calf to this second service after a temporary vaginitis.
	do.	C4	Washed Trichomonas into vagina immediately after service. Str. Utrecht.	Seen only at seven months for seven days but numerous during this period.	Infection established.	No significant change during course of experiment.	Slight vaginitis in first weeks, then acute vaginitis at six and a half months with Trichomonas. Condition subsides; healthy calf at full time to first service.
	do.	C7	Washed Trichomonas into vagina immediately after service. Str. Belfast.	Trichomonas only seen once at four months.	Infection established, probably very slight.	No significant change during course of experiment.	Slight vaginitis. Full-time calf to this first service. Calf found dead in field at seven weeks.
	do.	C8	Washed Trichomonas into vagina immediately after service. Str. Belfast.	Trichomonas seen for first fortnight, then at two months and five months.	Infection established.	Slight but not significant rise during course of experiment.	Vaginitis of varying severity during seven months but pregnancy continues–neither cow nor calf very healthy but both well a short time after calving.

normal pregnancy had followed. She had been treated with iodine and was perfectly healthy when used for this experiment.

(3) *Carry over infection by bull*

The cow C4 had been infected in the " C " series two years before but had been very thoroughly treated with iodine and had since shown no symptoms. The present infection was carried out by letting the hitherto uninfected bull serve cow (A8) into whose vagina a large number of living Trichomonas had just been introduced and then immediately afterwards serve C4.

(4) *Exposure of virgin heifers to infection at service by bull known to harbour Trichomonas.* Virgin heifers A2 and A3.

The results of these various methods are shown in Table II, and can be summarised as follows :—

(1) The attempt *via* the vagina in virgin heifers A4 and A6 produced no recognisable infection, the pregnancy was not interrupted and there was no change in the titre of the blood.

(2) The introduction of living Trichomonas into the uterus immediately after service gave rise to infection with temporary interruption of pregnancy, but after two returns the third mating was successful in A5 (the virgin heifer). A typical infection resulting in a state of " sterility " was produced by this method in cow C10. There was a slight rise in the titre of the blood in the heifer A5 but hardly sufficient to be significant. A clear rise in titre to the " positive " level was recorded in C10, which lasted three months and then declined to a borderline positive or definitely " above normal".

(3) The carry over of the Trichomonas by the hitherto uninfected bull from the cow with numerous parasites to the cow without parasites resulted in an infection and the breaking of service twice at six weeks. The titre of the serum rose to a borderline positive or definitely " above normal", and persisted at this level for five months.

(4) The infected bull transmitted no infection that could be recognised to the virgin heifer A3 ; there was a normal pregnancy from this service and the blood titre showed no change. The heifer A2, served one day later by this same bull, had a typical infection with numerous Trichomonas ; the service was returned once at 7 weeks and again at 12 weeks. There was, however, no change in this case in the titre of the serum. The state of this animal was particularly interesting and will be considered again in describing experiments in which it was subjected to a uterine instillation of Trichomonas extract.

One or two points should be noticed in connection with the experimental infections. The cultures used were not always strictly sterile but the contaminant seems not to have played any serious part in the result. The contaminant was a diphtheroid but not *C. pyogenes*. A serum test of the heifers of the A series kindly carried out by Dr. Lovell showed such very low titres for *C. pyogenes*

antitoxin that infection could be excluded. It should be particularly noted that symptoms of sterility, *i.e.*, the breaking of service, were never produced in any of these experiments except where Trichomonas was discovered by direct examination to have been present during some period long enough for it to be considered as an established infection. That is to say, these experiments with trichomonads from cultures have not produced any cases of sterility where *Tr. foetus* was not demonstrated. In addition, from the standpoint of this investigation into the serological response it was irrelevant whether the cultures were bacteria-free or not. All that was required was the setting up of the infection. The experimental transmission of *Tr. foetus* to cattle with pure cultures has been demonstrated already by Andrews and Miller (1938), Rees and Garlick (1939) and Abelein (1941).

II. ABSORPTION OF ANTIGEN

The direct infection experiments having produced many of the clinical features of the disease as seen in the field and also the characteristic early breaking of service and sterility, the correlation of these incidents with the ascertained blood titre still remained obscure. It had been demonstrated that a raised titre for the agglutinins against Trichomonas could be produced by experimental introduction of living organisms into the uterus but that active infection with subsequent breaking of service, etc., could also co-exist with a failure of serological response.

Uterine Instillation of Trichomonas Extract

In the next approach the antigen in the form of a sterile saline extract of *Tr. foetus* was instilled into the uterus of empty cows. (For the details of the preparation of the extract see Appendix II.) Owing to the exigencies of the times, the first extract experiment had to be done on borrowed or old cows about to be cast of whose history there was no accurate account. A normal blood titre was observed, however, before the experiments were started. There were difficulties in estimating the value of the extract and at first no convenient method of standardisation was available. Later the percentage of nitrogen present was used as a rough guide of potency. In the early experiments the method adopted was to make pooled batches and use one lot for a series of instillations and also to test the antigenic power of the extract intramuscularly in a spare animal.

B1 and B3 were treated with the same extract. A massive dose was instilled into B1 but no serological response was observed in the way of a raised titre. Three weeks later three smaller doses were given but there was no change in the titre and there was no generalised reaction in the animal. No *post-mortem* examination was made.

B2 received into the empty uterus 50 c.c. of extract repeated four days later and 25 c.c. three days after that. At this third dose a generalised reaction which will be described later in this section was produced. Four

TABLE II

Expt.	*Previous History*	*No. of Animal*	*Type of Infection*	*Trichomonas Findings*	*Result*	*Serological Findings*	*Outcome*
A. 1941-1942	Virgin heifer	A4	Washed Trichomonas into vagina after service.	Never seen.	No infection demonstrated.	No change in titre during experiment.	Transitory vaginitis. Pregnancy not interrupted; full-time calf but born dead.
	do.	A6	Washed Trichomonas into vagina after service.	Not seen.	No infection demonstrated.	Titre is on normal level throughout experiment.	Vaginitis about the fifth month; pregnancy not interrupted. Full-time healthy calf.
	do.	A5	Washed Trichomonas into uterus by means of catheter.	Trichomonas in large numbers for first six weeks, not seen after this.	Infection demonstrated during first six weeks.	Titre rising slightly for a time but not significantly.	Acute vaginitis with Trichomonas. Service broken at six weeks; mated, and again in season at three weeks; mated third time. Pregnancy is completed, healthy calf at full time.
	Healthy cow, one previous calf. (Served two years previously by bull unsuccessfully exposed to infection. No disease resulted but treated with iodine.	C10	Washed Trichomonas into uterus after service. Repeated after first return at three weeks.	Trichomonas seen in first three weeks. Then after re-infection Trichomonas seen at intervals for two months.	Infection demonstrated.	Titre rises to positive eleven days after first infection, remains high for three months, then is a borderline + or definitely "above normal."	Vaginitis acute. Service broken at three weeks; served again and re-infected vaginitis with Trichomonas; service broken at five weeks. Mated infected bull (third service). Return at five weeks; mated (fourth service), slight discharge. Trichomonas seen. Return at twelve days, not served again; treated with iodine.

TABLE II—*continued*

Expt.	*Previous History*	*No. of Animal*	*Types of Infection*	*Trichomonas Findings*	*Result*	*Serological Findings*	*Outcome*
	Young cow, previous infection with Trichomonas. Treated effectively with iodine.	C4	Carry over infection. Bull had immediately before served cow into whose vagina very numerous Trichomonas had been introduced.	Trichomonas very numerous at four to five weeks. Not seen again.	Infection demonstrated.	Titre rises to border-line positive and definitely "above normal." Persists at this level for five months.	Slight vaginitis, increasing at five weeks and showing Trichomonas. Service broken at six weeks. Mated infected bull. Discharge continues and vaginitis, Trichomonas not seen. Service again broken at six weeks; not mated again.
	Virgin heifer	A3	Served by infected bull 18.7.42.	Not seen.	No infection with Trichomonas demonstrated.	No change in titre.	A slight vaginitis, but no Trichomonas seen. Vaginitis subsides, normal pregnancy; healthy calf at full term.
	Virgin heifer.	A2	Served by infected bull one day after A3, 19.7.42	Trichomonas seen at six weeks after first service. Very numerous at eight weeks after second service.	Infection demonstrated.	No change in titre.	Slight vaginitis with Trichomonas seen at six weeks. Return of oestrus at seven weeks, served by infected bull. Copious discharge with many Trichomonas eight weeks after second service. Service broken at 12 weeks but not served again.

days later, *i.e.*, on the eleventh day since the first instillation, the serological response was marked and the titre had risen to a definite positive level. The index was 18/9 and all the characteristics were present of a positive agglutination of living trichomonas. Further instillation of 15 c.c. four days later (15th day of the experiment) produced a repetition of the generalised symptoms but only to a very mild degree. The final 20 c.c. instilled on the 18th day produced no reaction. The titre of the serum was maintained at the high level but was not increased by these later instillations after the eleventh day. There was no *post-mortem* examination.

In B3 the extract was injected intramuscularly as a control of its antigenicity. A total of 60 c.c. was used in four doses and a characteristic rise in the titre to the positive level was observed.

B4 and B5 were treated together on parallel lines receiving 180 c.c. in five doses of the same sample of extract. B4 showed no generalised systemic reaction. The blood titre only rose to a borderline definitely " above normal " level but did not reach the actual positive standard. At *post-mortem* examination the uterus was found to be in an inflamed condition with thickened walls and petechial haemorrhages. B5 also showed no systemic reaction. The serological response was definite and a high positive titre for living trichomonas was produced. *Post-mortem* examination showed nothing abnormal in the appearance of the uterus.

Four more cows whose previous histories were unknown were treated by instillation of extract into the uterus. Three of them showed systemic reactions but never with the first dose exhibited, and all three developed definitely positive agglutination titres in the serum. The fourth animal (B9) responded sluggishly, showing a delayed and not very good serological response ; the titre, while reaching the positive level, never rose as high as in the other animals. There was a slight systemic reaction at the fourth dose which was a massive one.

When we consider these " B " experiments within the limitation of the absence of full knowledge regarding the previous history of the animals we can divide them into two groups : (1) those which were non-receptive (B1 and B4), where there was no serological response or only a very slight one (these did not show any systemic reaction), and (2) those which were receptive. The receptive animals were six in number. All these sooner or later responded with a clear cut rise in the titre of the agglutinins against Trichomonas to the positive level. Five out of the six developed the generalised systemic reaction but in different degrees. One animal (B5) showed no recognisable systemic reaction although there was a good rise in the agglutination titre. This series demonstrated that the antigen could be absorbed by the uterine route and that antibodies could consequently be formed in the blood, but that in certain circumstances there was no response, *i.e.*, the antigen failed to be absorbed or at all events the agglutination titre was not enhanced. It is admitted that while the evidence is very good, it is not definitely demonstrated here that sensitisation has occurred in animals which have never come into contact with *Tr. foetus*. It would, however, be extremely unlikely that five out of six apparently receptive cows which were sensitised should all have had a previous infection which

had disappeared without leaving any trace. This matter is being further investigated.

Systemic Reaction and Sensitisation.—The systemic reaction mentioned above when produced by instillation into the uterus of saline extract of trichomonas appeared at the earliest in 30 minutes and at the latest in one hour after the instillation. During the interval, pulse, respiration and appetite were not affected. The first symptom to be noted was that the animal showed no desire to move about; this was followed by cessation of feeding and an increase in both the pulse and respiration rate. The pulse rate might reach 60 to 80 per minute and the respiration 30 to 50. No rise in the temperature was observed in any of the cases. Tympanites, as shown by the distension of the flanks gradually increased until in certain cases, as, for example, in A10 and B8, where the reaction was very severe, a tense drum-like condition developed with the additional secondary symptoms of anxiety and respiratory distress. The rapidity of the recovery depended on the severity of the attack; in some cases the animals returned to normal in about an hour, while in others it took eight to ten hours for the symptoms to pass. It was found that subcutaneous injections of 3 c.c. of adrenalin produced early alleviation of the symptoms of distress, principally that of tympany. This improvement took place in 30 minutes, suggesting the relaxation of the involuntary muscle fibres. It is probable that the tympany is induced by the contraction of the involuntary circular muscle at the opening of the rumen.

The systemic response which is interpreted as a sensitisation of the animal to the antigen does not necessarily take place during the production of the antibody in the serum. That is to say, animals can produce antibody without being sensitised; they can be "immunised" in this sense without sensitisation. But no example was found of a systemic generalised reaction without a substantial rise in the titre; that is, a display of sensitisation was not evoked unless antibody was also produced by instillations into the uteri of these animals believed to be normal. This point is not stressed here; it is merely stated as an observation of fact. It is most probably not of universal occurrence in natural infections as is shown later on by the reactions of an animal, A2.

The problem of sensitisation is not clear as yet and further work is needed to understand its mechanism and what part, if any, it plays in the disease in the field. It was encountered as an accompanying phenomenon while investigating the means whereby the enhanced titre was acquired. In the next set of experiments it appeared again and while, as already stated, its role is not fully understood its occurrence, nevertheless, threw a good deal of light on the state of some of the animals.

Instillation of Extract of Trichomonas into Cows of Known History

The instillation method using the sterile extract of Trichomonas was extended to a group of animals about whose history detailed knowledge was available.

A1 (normal control to animals of the A series), virgin heifer,

had been served (1941) by a young, clean bull and had a healthy full-time calf. Instillations of extract into the uterus were begun 23 days after calving. The extract was active, as shown by the behaviour of other animals treated with the same batch.

The injections into the uterus were as follows : 16.4.42, 25 c.c. ; 23.4.42, 25 c.c. ; 29.4.42, 50 c.c. ; 18.5.42, 100 c.c. The titre rose 12 days after the first instillation to a moderate positive (index $15\frac{1}{2}/9$). There was no systemic reaction ; the titre of the serum did not rise higher. *Post-mortem* examination showed nothing abnormal in the uterus. There was a rather poor serological response and the animal was apparently not sensitised.

A2 (see Table II). This animal had been successfully infected with Trichomonas and rendered sterile. It was not treated with iodine but was allowed to rest without further service for three months and three weeks before instillation of extract. (The same batch was used as for A1.)

The details are as follows :—

On 17.4.42, 25 c.c. was instilled into uterus and a pronounced systemic reaction was observed in one hour. On the sixth day after this first instillation the titre rose to a moderate positive (index $15\frac{1}{2}/9$).

On 23.4.42 25 c.c. was instilled into uterus and was followed by a mild systemic reaction. Six days later, on the 12th day, the agglutination titre was a high positive (index $19\frac{1}{2}/9$).

On 29.4.42, 50 c.c. was instilled into uterus ; there was a severe systemic reaction.

A2 was afterwards treated with iodine.

The important point here is that the animal was clearly in a sensitised state at the first instillation and it seems reasonable to conclude that this had been brought about by infection with Trichomonas during the previous months. It should also be noted that A2 was in a sensitised state although there had been no evidence of a raised agglutination titre during the course of the infection. The high titre produced by the instillation lasted for at least 28 days but had declined to a borderline positive or only a definitely " above normal " titre 57 days after the last dose. It had further decreased to a just recognisable degree above normal in 85 days, at which state it remained. A1 and A2 are controls of each other. A1 was apparently not a good producer of antibody, and although a high titre was reached in A2 its duration was relatively short.

C4 and *C10* (see Table II). Both these animals had been successfully infected in 1941 and had shown symptoms and become sterile ; both were adequately treated with iodine in October and November, 1941. They had above normal agglutination titres with an index of $12\frac{1}{2}/9\frac{1}{2}$, that is, they were borderline but not true positives just before the instillations. The first dose produced no generalised symptoms in either case and only at the third dose was there a moderate systemic reaction, which was of similar intensity in the two cases. These two animals, therefore, had not been sensitised by the previous infection but did become sensitised to a moderate degree during the course of the instillations. The serological response was

definite and the titres rose to the true positive level although C10 responded better than C4. This latter animal did not produce a very high or lasting agglutination titre although it responded for a time. The serological findings during infection (see Table II) had also been less definite than with C10.

A10 is an example of a field infection. She had been bought from a farmer after she had aborted at three and a half months showing a copious discharge with many Trichomonas. The parasites could be demonstrated for about three months after the abortion, at which period the cow was successfully treated with iodine. The blood titre was at the level of a high positive when the animal first came under observation ; it remained high and was at this level just before the instillation of extract was carried out five months after the treatment with iodine ; 25 c.c. of the same batch of extract as that used for the four animals just described above were instilled into the uterus. There was acute systemic reaction within 30 minutes of carrying out the instillation. Three weeks later a smaller dose of 20 c.c. of the same extract provoked no generalised reaction. This animal had been sensitised apparently by a natural field infection. In this case the agglutination titre was high at the time of the instillation which provoked the reaction.

High Titre in Calves

The interesting question is being studied as to how the high titre is acquired which has been observed in calves of cows whose blood contained a raised agglutinin titre for *Tr. foetus*. There are practical difficulties in the way of this work but one very clear instance has been obtained (B14).

An extract of Trichomonas was injected intramuscularly into a pregnant cow 33 days before she was due to calve. She was given four injections totalling 70 c.c. After the third dose the titre had risen to the level of a high positive and the index was $19\frac{1}{2}/9$. The animal calved 21 days after the fourth dose, at which time the titre had dropped to 15/8. The calf was removed and bled *at the moment of* birth ; when 15 minutes old it was given three pints of the cow's colostrum. One hour later the calf was bled again. Samples were also taken of the cow's colostrum.

The results can be summarised as follows : the cow's titre remained at 15/8 for eight days after calving, when she had to be returned to her owners. Whey from the colostrum at the birth of the calf showed a definitely higher titre for *Tr. foetus* than that of the cow's serum, the index being $18\frac{1}{2}/8\frac{1}{2}$ and it remained at this high concentration for 48 hours showing only a very slight diminution. The titre of the colostrum was at its peak at 16 hours, reaching $19\frac{1}{2}/8\frac{1}{2}$. The calf at birth showed no agglutinins whatever in its serum against *Tr. foetus*, but at 75 minutes old, that is 60 minutes after its first feed, agglutinins were present in a concentration which

gave an index of 5 to the normal of $7\frac{1}{2}$ for an adult for the day. The serum of the calf from the sample at 16 hours had a very high titre, the index being $19/7\frac{1}{2}$, only very slightly lower than that of the whey at the same period and quite definitely above that of the maternal serum. At the age of 17 days the calf was still a high positive but the titre was just appreciably lower than on the first day.

While this is very clear evidence that the calf can absorb the maternal antibody directly from the colostrum through the alimentary canal and is an example similar to that found by Mason, Dalling and Gordon (1930) in the sheep, it does not exclude the possibility of the passage *in utero* also taking place under slightly different conditions. The writers are not quite convinced that this does not occur and find the very long persistence of high titres in certain calves difficult to account for as a purely passive transference by the ingestion of the colostrum. The matter is receiving further study owing to its importance in diagnosis and possibly also in the resistance to the disease. It is not proposed to go into greater detail in considering the bearing of this experiment at the moment.

DISCUSSION

In the infection experiments recorded above, a great many of the clinical features and types of the disease found in the field have been reproduced and their relation to the production of agglutinin studied. The practical importance of the low, almost subclinical, infections evoked in the " C " group (see Table I) cannot be overestimated. They cannot be traced with any certainty by means of the agglutination test ; direct examination may easily fail to detect the organism as in practice the small number of examinations may not coincide with a period of multiplication. These low infections are not serious on account of their immediate economic loss for that may be trifling in the early stages of the outbreak. Their importance lies in the harbouring of the parasite, the possible infection of the bull and the fostering of unknown reservoirs of infection. From these undetected sources the infection may be introduced into valuable self-contained herds where it gradually in course of time might produce the familiar serious outbreaks with sterility and abortion.

The " A " experiments (see Table II) have shown the next grade of infection where the clinical symptoms are much more obvious, where breaking of service and failure to conceive and in fact the sterility picture and the states leading up to it were reproduced. The agglutination titre rose in certain animals to the truly positive level and in another case only became " above normal", but in this group also an animal was found which in spite of characteristic symptoms and certain infection failed to react serologically in any obvious way that could be detected by the direct agglutination

test (A2). In this group some animals resisted infection, showing the fairly high normal resistance of cattle to this disease. The observation of the infected bull serving two heifers on consecutive days which resulted in one becoming infected and the other not should be noted, as it is known to occur in field cases, but it is not an argument against the venereal and infectious character of the disease.

The means by which the antigen is absorbed is shown to be from the uterus and these experiments revealed two main sources of failure in the production of circulating antibody : first, the failure to absorb the antigen from the genital tract and, secondly, the failure to react by the production of antibody in sufficient quantity to be detected by the methods used. The failure to absorb, at least in certain cases, is due apparently to the existence of inflammatory processes in the uterus. But apart from this some animals appeared to be poor antibody producers, a phenomenon well known in other types of immunological response.

The interesting sensitisation reactions observed are apparently anaphylactic phenomena suggesting the cell fixation of the antigen probably in the smooth muscle of the rumen and possibly of the uterus, but its importance, apart from the light it throws on the absorption of antigen and the evolution of antibody, is not yet clear.

The control and reproduction of the phenomenon is not yet sufficiently developed and its discussion in greater detail is premature. That it may play a part in the actual production of abortion is obviously suggested but it is not demonstrated at present. The experiments show that the systemic reaction is not immediately related to the presence or absence of a high agglutinin titre in the serum of the animal. It would appear from the nature of the extract that protein other than that contained in the Trichomonas itself is not involved in the reaction. It should be noted that sensitisation had been produced in A2 by an infection which had evoked the local symptoms, but which had failed to show the characteristic rise in the blood titre and also that A10 had been sensitised by a natural field infection which had resulted in a typical abortion with the development of a high blood titre. There is nothing surprising in this as the essence of the sensitisation would appear to be the cell fixation of the antigen, not the circulating antibody.

It will be observed that the experimental infections with living organisms have not so far produced the characteristic abortion at two to four and a half months with Trichomonas in the amniotic fluid and in the alimentary tract of the foetus. This type of frank abortion is in any case by far the least frequent and most acute manifestation of the disease. Infection of this kind has been reproduced, however, by Rees and Garlick (1939).

The value of the agglutination test in the study of trichomoniasis has been obvious in the course of this work, but its limitations have also been clearly shown. The arbitrary positive level was originally

chosen on technical as well as experimental grounds because of its sharp and objective distinctiveness. This choice has, moreover, received support from the later work which has revealed the composite nature of the group of animals showing the intermediate titre, that is those below " positive " but " above normal". The positive sera characterised by the pre-agglutination zone and/or the flat échelon form of clumping have again been correlated with a serious degree of infection or with the absorption of Trichomonas antigen. It has been found, outside these animals, only in the sera of very young calves of animals possessing a high titre of circulating antibody.

The " above normal " group with the intermediate titre has been found to consist of (1) animals which have had a true positive titre that is in process of decline, that is to say, they are animals with fading titres ; (2) animals which are or have been infected with Trichomonas, but whose absorption of the antigen has been inadequate because of the condition of the genital tract ; (3) animals where the antigen has been absorbed but whose antibody mechanism has not reacted vigorously enough to produce a true positive level of agglutinin ; (4) animals with moderate or low infections not sufficiently acute to produce the full serological response ; (5) animals carrying from birth " above normal " or slightly irregular titres acquired in some way from the parent. An example of the absorption of the antibody from the colostrum has been given.

This investigation has increased the clinical significance of an " above normal " titre, but the existence of the raised titre which can be passed to an animal not itself in any way infected and which has not been in direct contact with the infection, makes the appraisement of the significance of the animals with this intermediate reaction somewhat difficult and uncertain. Where several animals with " above normal " titres, but not at the positive level are found in one herd, further investigation will frequently result in the finding of one or more individuals with an unmistakable raised titre and/or the demonstration of the parasite by direct examination. The duration of the raised titre is very variable and so far no explanation of this is forthcoming. Again, it is a phenomenon often met with in infections and in the immunisation or vaccination of man and animals with various organisms.

SUMMARY

(1) A study of the antibody response of cattle to the antigen of *Trichomonas foetus* has been made by means of the agglutination test.

(2) The artificial infection of virgin heifers with washed cultures of living *Tr. foetus* was carried out by the introduction of the protozoa into the vagina after mating. The infections were successful but pregnancy was not interfered with in four out of five animals. The fifth animal returned at three weeks and was served six weeks later, producing a successful pregnancy with a healthy calf at full term.

This low type of infection caused no change in the agglutination titre of the sera of these animals.

(3) The introduction of living *Tr. foetus* into the uterus after service gave rise to various degrees of disease closely comparable to infections found in field cases. Certain of these animals showed a quite definite rise in the agglutination titre, but the degree of blood response varied and one animal, although rendered sterile, did not manifest any change in the antibody content of the serum.

(4) A saline extract of *Tr. foetus* was instilled into the uterus of non-gravid cows. This resulted in most (but not in all) cases in the absorption of the antigen and the production of an enhanced agglutinin titre in the serum.

(5) The degree of antibody response consequent upon the instillation of extract varied and this appeared to be due to two causes : (i) the non-absorption of the antigen, and (ii) the failure to respond to the stimulus by the production of antibody.

(6) The instillation of extract of trichomonas produced in many cases a systemic reaction, usually after the third dose, due to the sensitisation of the animal to the extract.

(7) This state of sensitisation was found to be present upon the first instillation of the extract in one animal—a field case which had aborted nine months previously and which had harboured Trichomonas for at least six months.

Sensitisation was also found in another case as the result of a successful uterine infection with living culture. In this instance, although symptoms had been clear and the parasite had been demonstrated over a period of five months, the blood titre had not risen. This animal was sensitised by the infection without producing a change in the agglutinin content of the blood.

(8) The calf of a cow possessing a moderately high blood titre was found to have acquired a high titre 16 hours after having been fed with the maternal colostrum. The colostrum showed a concentration of agglutinin slightly above that of the maternal serum. At birth, before being fed, the calf's serum contained no demonstrable agglutinin for *Tr. foetus* in a dilution of 1/6, but after having ingested the colostrum the titre of the calf's serum was at the level of the colostrum and definitely above that of the maternal serum.

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APPENDIX I

The Agglutination Test for Trichomonas Foetus.—The following brief notes will facilitate the understanding of the references to the test in the text for those who have not made themselves familiar with the earlier description.

The sera must be free from preservatives and as nearly sterile or at least as clean as possible. They are always heated to 56° for 15 to 20 minutes. This is important as protozoa are extremely sensitive to the presence of even slight traces of complement. A living suspension is used. The dilutions which proceed by multiples of two as usual, begin with serum at 1/3 which in the final state after adding the suspension amounts to a dilution of 1/6 in the first plate and can be carried out to 1/1536 if desired. The titre must always be considered when evaluating unknown sera in the light of the normal control of the day.

A simple method of doing this by means of the conventional marking has been devised.

The pre-agglutination zone characterised by immobilisation, the formation of small leaf-like clumps or even the absence of clumping among the many immobilised organisms, a shrinkage of the body of the organism and in some cases their death in 22 hours. *Marked with* 5 *crosses.*

Flat échelon leaf-like clumping usually one cell thick with much reduced motility and with signs at 22 hours of the shrinkage of the bodies. *Marked with* 4½ *crosses.*

Big round clumps giving a completely clumped-up appearance with the flagella of the Trichomonas usually still motile. *Marked with* 4 *crosses.*

Similar clumping with, however, free organisms very few in number and a greater degree of motility. *Marked with* 3½ *crosses.*

Well clumped-up with a few free organisms. *Marked with* 3 *crosses.*

Big and smaller clumps giving a clumped-up appearance with a good number of free organisms. *Marked with* 2 *crosses.*

A dispersed suspension with good clumps in it. *Marked with* 1 *cross.*

Small loose aggregates in good numbers and small clumps in a well dispersed suspension. *Marked with* ½ *cross.*

Intermediate types between 3 and 2 and 2 and 1 are designated 2½ and 1½.

The ratio of normal to unknown and positive sera can be conveniently expressed by counting the crosses given in marking *the first four plates* of

the series and placing the figure in the unknown over that of the known normal. This is used in the text and is called the " index."

Normal heated sera in tests beginning with dilutions at a final computation of 1/6, a lay-out that is always adhered to, usually show good agglutination marked with three to three and a half crosses in this concentration. They do not show échelon flat agglutination (E) nor the pre-agglutination (P.A.) appearances. This has been made the arbitrary distinction between " positive " sera and normals and above normals. " Above normals " have a higher titre than the normals with the same suspension on the same day, but do not show the characteristic échelon flat clumping or the pre-agglutination state.

The numbers of organisms in the suspension should be about 100 per cubic c.c. when added to the sera. The low numbers make for greater precision in the readings which are carried out under the low powers of the microscope.

APPENDIX II

Preparation of the Extract of Trichomonas.—*Tr. foetus* is grown in 20 oz. bottles of G.B.S. medium, *i.e.*, inspissated bovine serum and glucose broth 2 per cent. covered with liquid paraffin. At approximately the fourth day when growth is very heavy the broth is centrifuged at high speed in 100 c.c. buckets. The sediment is then washed in sterile saline and again centrifuged. To the sediment (according to quantity) sterile distilled water is added and the mixture allowed to stand for one hour, by which time the laking of the bodies is complete. An extracting solution of 0·5 per cent. NaCl, 0·25 per cent., sodium bicarbonate and 0·4 per cent. phenol is added to the laked suspension. The quantity added is the same as that of the distilled water, therefore the final quantities are halved, that is, salt 0·25 per cent., sodium bicarbonate 0·125 per cent. and phenol 0·2 per cent. After four days' extraction, the fluid is filtered through a sterile Seitz filter pad, bottled (in dark bottles) and stored in the refrigerator ready for use. Chemical analysis by Dr. Common shows 0·8 per cent. nitrogen.

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CORYNEBACTERIUM EQUI IN THE SUBMAXILLARY LYMPH NODES OF SWINE

By

E. Cotchin,

Research Institute in Animal Pathology, Royal Veterinary College, London

INTRODUCTION

In a stud of thoroughbreds in Sweden, Magnusson (1923) drew attention to a specific infectious pneumonia of foals caused by a gram-positive diphtheroid to which he gave the name *Corynebacterium equi*. Later (Magnusson, 1938) he gave a further account of the disease, of which he had seen, between 1917 and 1931, 41 cases, all except one occurring in the same stud. Since 1931 the practice was adopted for the mares of this stud to foal on premises other than those on which cases of infection had occurred; the disease then disappeared. The infection appeared mainly in foals from two to three months old, and was easily differentiated from strangles or joint-ill by the absence of lesions in the cervical lymph nodes and the joints. In most cases there was a suppurative broncho-pneumonia, with large abscesses in the lungs and intense purulent inflammation of the pulmonary lymph nodes; in some animals the mesenteric lymph nodes were also involved, and the intestinal wall was ulcerated. Magnusson (1938) gave the following as the main characters of the organism, and stressed the difference between it and *C. pyogenes*, with which organism it had at times been confused.

"Morphology: Obviously gram-positive, large bacteria. In pus, ovoid or coccus-like. In superficial cultures, resemble streptococci. In broth and in deep colonies, irregular rods, lying in V-formation, sometimes branched like streptothrix.

"Cultures: Optimum temperature 18° to 37° C. Abundant growth on all ordinary media, with large succulent or mucous colonies of irregular shape, which assume a pale pink colour. In serum agar, large colonies are visible already after one day, as vigorously developed at 20° as at 37° C. On potato, moist thick growth, which soon takes on a pale pink colour, and afterwards turns a deep reddish yellow. Poor growth in milk, no coagulation. . . . No haemolysis. . . . Does not affect any fermentable substance."

Since Magnusson's first article appeared, the disease has been reported from several countries; it is usually fatal, but the number of cases recorded is not large, as the condition tends to occur in small outbreaks, mainly in the larger breeding establishments. *C. equi* infection of foals has been recognised in many parts of the Continent (Magnusson, 1938), in India (Rajagopalan, 1937), in Australia (Bull, 1924), and in America (Dimock and Edwards, 1931; Dimock, 1941). The fact that the disease has not been recorded in foals in Great Britain, even following the very clear description brought before the practitioner here by Magnusson (1938), makes it probable that if it exists at all in this country it is only to a very limited extent. However, *C. equi* was recently found to occur in England by Craig and Davies (1940), who isolated it in pure culture from the uterine discharge of two cows affected with pyometra. An analogous finding is that of Rajagopalan and Gopalakrishnan (1938) in India, who recovered the organism from the cervix of a she-buffalo that had recently aborted. Strains of *C. equi* have

also been isolated from the os uteri of barren mares and from aborted equine foetuses (Bruner, Dimock and Edwards, 1939).

In 1936, Holth and Amundsen, working in Eastern Norway, recovered a " specific acid-fast cocco-bacillus " from suspected tuberculous lesions of the cervical lymph nodes of swine. It is probable, but not certain, that this organism was *C. equi*, as other workers in Scandinavia have since reported the recovery of *C. equi* from such lesions (*e.g.*, Plum, 1938, 1940 ; Magnusson, 1938, 1940 ; Bendixen and Jepsen, 1938, 1940), and it has been found in suspected tuberculous lesions in France and Switzerland (Verge and Senthille, 1942), in America (Karlson, Moses and Feldman, 1940), and in Australia (McDonald, 1942).

Since it is well recognised that it is not always possible to demonstrate viable tubercle bacilli in tuberculous-like lesions of the throat glands of swine, it seemed possible, in view of these findings, that *C. equi* might be the cause of some, at least, of those lesions from which tubercle bacilli could not be isolated. Conflicting views have been expressed by those who have studied this subject. Thus, Bendixen and Jepsen (1938) consider that infection of the cervical lymph nodes of swine by *C. equi* is probably a definite pathological entity ; they describe the typical lesion as consisting of small discrete yellowish encapsulated spherical nodules, frequently occurring just beneath the capsule of the node. Plum (1940), on the other hand, as a result of experiments on swine, considers the organism likely to be of importance only as a secondary invader. A third view is that of Feldman, Moses and Karlson (1940) who believe that the organism is not aetiologically related to tuberculous-like lymphadenitis of swine, as they found it as commonly in normal as in abnormal lymph nodes, and could not reproduce the condition in pigs by feeding them with cultures.

No records exist of the isolation of *C. equi* from tuberculous-like lesions of the throat glands of swine in Great Britain, and the question of its occurrence is of more than academic interest in its relation to meat inspection. An investigation has therefore been made to determine whether *C. equi* occurs in pigs in this country, and with this object in view, an examination has been made of lymph nodes showing macroscopic lesions, apparently tuberculous in nature, and of nodes which appeared normal. The further problem of the pathogenicity of *C. equi* for swine has been studied by administration of suspensions of the organism to pigs. It was thus hoped that an opinion might be formed as to whether *C. equi* is associated aetiologically or merely incidentally with tuberculous-like lesions of the cervical lymph nodes.

MATERIAL AND METHODS

C. equi and the tubercle bacillus resist an exposure to oxalic acid that will kill many other bacteria. For example, Karlson, Moses and Feldman (1940) exposed strains of *C. equi* for 15, 30, and 60 minutes, to 1·5, 2·5, 3·75, and 5 per cent. concentrations of oxalic acid, and obtained cultures subsequently in all cases ; similar treatment killed staphylococci, streptococci, *Bact. coli*, *B. subtilis*, and a diphtheroid producing yellow colonies. Thus, if

C. equi and tubercle bacilli are present in contaminated material, treatment of this material with oxalic acid should facilitate their isolation, although an obvious disadvantage of the method is that any other organism that might be of significance would probably not be recovered. It is also possible that if *C. equi* was present in small numbers it might fail to develop in culture after exposing the material to oxalic acid.

EXAMINATION OF LYMPH NODES SHOWING TUBERCULOUS-LIKE LESIONS

One (27 pigs) or both (59 pigs) submaxillary lymph nodes, showing macroscopically what seemed to the meat inspector to be typical tuberculous lesions, were taken from 86 pigs, and transported separately in sterile bottles from a bacon factory to the laboratory. Each gland was there picked out from its container with sterile forceps, plunged directly into boiling water, and held there for several seconds with the object of destroying as many as possible of the surface contaminants. The gland was next cut up finely with sterile scissors, and the pieces were ground up in a mortar, at first alone, and then with a little saline. The resulting thick suspension was transferred by a wide-bore pipette to a sterile tube. In the case of the glands (17 single, 19 pairs) from the first 36 pigs, the procedure was then as follows : to the thick suspension in the tube was added an equal volume of a sterile 5 per cent. w/v solution of oxalic acid crystals in water, giving a final concentration of 2·5 per cent. The tube was shaken at intervals to ensure mixing, and after 5, 15, and 20 minutes, about a dozen drops of the suspension were seeded directly, without neutralising, over the surface of plain egg slopes. The tubes were incubated at 37° C., and no tube was discarded as negative for at least four months. Some of these tubes remained sterile, and it was decided to include animal inoculation in dealing with the next series of glands, to see whether the number of positive results might be increased by this means. Accordingly, in the case of the glands (10 single, 40 pairs) from the next 50 pigs examined, one-half of the ground-up suspension was dealt with as above, while the other half, or a pooled sample where two glands had been taken from the same pig, was used for guinea-pig inoculation. About 1 c.c. of the suspension in each case was injected subcutaneously into the thigh of each of two guinea-pigs ; one animal from each pair was killed about a month later, and smears and cultures made from the local lesion, if present, and in all cases from the local gland and the spleen ; the other guinea-pig, unless it died earlier, was killed after three to four months, and examined in the same way.

The results obtained are shown briefly in Table I.

Strains of *C. equi* were isolated from the tuberculous-like lesions of the submaxillary lymph nodes of 8 of 86 pigs. There was no outstanding difference in the macroscopic appearances of those

TABLE I

RECOVERY OF *C. equi* AND TUBERCLE BACILLI FROM LESIONS IN THE SUBMAXILLARY LYMPH NODES OF 86 SWINE

	No. of Pigs
C. equi recovered alone	3
Tubercle bacilli recovered alone	63
C. equi and tubercle bacilli both recovered	5
Neither *C. equi* nor tubercle bacilli recovered	15
Total ...	86

glands from which *C. equi* was recovered, and those from which it was not isolated, as they showed various degrees of enlargement, and caseous and sometimes calcified foci of varying size. From three pigs, *C. equi* was isolated alone, while from five pigs, tubercle bacilli as well as *C. equi* were recovered. In four of these five cases tubercle bacilli were recovered from the inoculated guinea-pigs, but not in direct culture from the submaxillary gland lesion; in the other case, tubercle bacilli were not recovered from the inoculated guinea-pigs, but colonies of tubercle bacilli appeared in the culture tube inoculated after 20 minutes' treatment with oxalic acid, the colonies of *C. equi* appearing in the tubes inoculated after 5 and 15 minutes only. A more detailed account of the recovery and characters of the strains of tubercle bacilli isolated is given elsewhere in this issue. (Cotchin, 1943.)

EXAMINATION OF LYMPH NODES FROM NORMAL PIGS

The left submaxillary lymph nodes, all of which were apparently normal, were taken from a further 50 pigs at another bacon factory, and were examined for the presence of *C. equi* only, as follows: the nodes were ground up and treated with oxalic acid as before, and the suspension was seeded directly, without neutralising, over plain agar slopes, after 10 and 20 minutes' treatment. The tubes were incubated at 37° C., and no tube was abandoned as negative before two months had elapsed. *C. equi* was recovered from one of these glands (strain number 9); this gland was normal macroscopically, and paraffin sections did not reveal any microscopic lesions.

DESCRIPTION OF THE STRAINS OF *C. equi* OBTAINED

When freshly isolated, there was a considerable time lag before the strains gave a recognisable growth on inoculated media. The interval between the inoculation and the appearance of well-developed colonies varied from 13 to 37 days. A similar time lag was sometimes noticed when the organism was recovered from experimentally inoculated animals. Primary cultures should therefore not be discarded as negative for at least five weeks. This time lag is apparently a feature of the growth of the organism in primary culture from animal tissues, and not due to the use of oxalic acid, since on subcultivation, the strains grew quite as readily in 24 hours at 37° C.

in the presence of the strength of oxalic acid used for primary cultivation as in its absence.

Morphology.—There is a very variable morphology, so that individual organisms on the same medium and on different media differ widely from each other in appearance. They may be coccobacillary, bacillary, or filamentous in shape, tending to be shorter on solid media, where they may resemble cocci. In broth, the diphtheroid character is sometimes pronounced, and many examples of apparent branching may be seen. No capsules, flagella, or spores can be demonstrated. In very old cultures (four months) many diverse forms are seen, some short, some long, and some ring-like. The organism is in general gram-positive, but in all cultures, especially when old, many gram-negative forms are seen. Acid-fast forms, as described by Bendixen and Jepsen (1940) in films stained by the Ziehl-Neelsen method, with picric acid or malachite green as the counterstain, have not been observed.

Growth Characters.—The organism is an aerobe and a facultative anaerobe, and grows in an atmosphere containing up to 40 per cent. CO_2. Growth occurs almost as readily at room temperature as at 37° C. When grown on agar plates for 24 hours, the colonies are amorphous, rounded, with an entire edge, and pale pink in colour. The surface is smooth and glistening, and the structure is homogeneous. After a few days the colonies are up to 1 cm. in diameter, the colour deepening to a rich salmon-pink. The edge of the colonies tends to become rather clear and watery-looking with age, the centre remaining opaque. The colonies are so moist that portions may fall in the form of drops on to the lid of the Petri dish, and a platinum loop may sometimes be drawn through a colony without disturbing its shape. Individual colonies tend to fuse together, giving an appearance of a continuous streak of growth. A similar picture is obtained with agar slopes, on which the growth runs to the bottom of the tube, forming there a thick pigmented layer. Good growth occurs in MacConkey agar. Haemolysis is not produced in blood agar plates, on which the organism grows readily. When cultured on egg slopes, there is a slightly wrinkled, rather drier growth, brick-red in colour. A good growth with rich pigment formation also occurs on potato slopes. Broth cultures show slight or no turbidity, occasionally a surface pellicle, and an abundant pink deposit; this deposit in young cultures is easily brought into suspension, and soon settles on standing, but in older cultures it appears to be more viscous.

Biochemical Reactions.—All the strains have been grown in peptone water with different carbohydrates and Andrade's indicator for 28 days at 37° C., but none of the following are fermented: glucose, maltose, mannite, lactose, saccharose, salicin. Litmus milk is unchanged; ammonia is produced, but no indole is formed, in peptone water; the Methyl Red and Voges Proskauer tests are negative; when tested by the ferric chloride method, the sodium hippurate

test is positive. The strains reduce 1 : 20,000 methylene blue, and form small amounts of H_2S when grown on lead acetate agar. Gelatin is not liquefied when stab cultures are incubated at 18° to 20° C., and no signs of liquefaction appear when the organism is grown on solid serum at 37° C. Black moist colonies develop readily on blood agar containing 1 : 500 potassium tellurite. The catalase test is positive.

Resistance.—The organisms remain alive for several months on plain media in sealed tubes. Their resistance to heat was tested by immersing suspensions, with an opacity of 2 as measured by Brown's standard opacity tubes, in a water-bath at 60° C. and at 80° C. Subcultures were made every five minutes by taking a few drops of the suspension for inoculating tubes of broth ; incubation of the tubes showed that, under these conditions, 60° C. was lethal in 60 minutes, and 80° C. in 10 minutes. Suspensions in normal saline (opacity, Brown 2) of the strains isolated, were acted upon by a final concentration of 2·5 per cent. oxalic acid, and several drop of the treated suspensions were seeded directly on to plain agar slopes every five minutes ; incubation of these showed that the strains resisted 2·5 per cent. oxalic acid treatment for at least 60 minutes. Resistance to 15 per cent. sulphuric acid varied, but in general this was not lethal unless allowed to act for 45 minutes ; it was noted that a 24-hour culture of one strain did not survive treatment for 40 minutes, whereas a two-months-old culture survived similar treatment for 50 minutes. The view that the resistance of *C. equi* increases with age has also been advanced by Bendixen and Jepsen (1940), who consider that more resistant acid-fast-staining forms develop in older cultures. However, no acid-fast forms could be seen in smears from the particular two-months-old culture tested here.

Serological Reactions.—Bruner and Edwards (1941) have examined the antigenic structure of *C. equi.* They produced antisera by intravenous inoculation of rabbits with killed suspensions, usually giving 12 doses, varying from 1 c.c. to 10 c.c. over a period of 30 days. Agglutination was found to be highly type-specific, but by using heat-acid extracts, precipitin tests were found to show group-specific antigens. An attempt has been made to repeat this work, but the results so far obtained have been disappointing. Four of the strains isolated from the tuberculous lesions were used. Each strain was injected intravenously into a rabbit, using suspensions of 24-hour agar slope cultures, killed by exposure to 0·3 per cent. formalin for eight hours at 37° C., in doses increasing by increments of 5 mg. from 1 mg. to 50 mg. at intervals of four to five days. One of the rabbits died after five doses, and another after eleven doses, after showing considerable wasting ; in neither case was *C. equi* recovered from the organs after death. The other two rabbits received 14 doses, but their serum gave no agglutination with heterologous strains (suspensions, opacity 2, of 24-hour agar slope cultures,

with 0·3 per cent. formalin added), and only slight agglutination at a titre of 1/10 with the homologous strains. The same four strains were used in the next series of four rabbits, which received formalin-killed suspensions intravenously, on each of four successive days in succeeding weeks. The daily doses in the first week were 6 mg., in the second week 24 mg., and in the third week 6 mg. One of these rabbits died at the end of the second week, having shown wasting, but *C. equi* was not recovered from it. The serum from the other three rabbits was tested after the third week of inoculations, but with the same negative results as in the case of the first series of inoculations.

It was then decided to employ living suspensions for inoculation, in view of the poor response to dead bacteria, and in this case, living suspensions of two known strains of *C. equi*, originally isolated by Magnusson, were used. Each strain was grown on an agar slope at 37° C. for 24 hours and the growth was then washed off and suspended in 1 c.c. of saline ; the rabbits each received doses intravenously at intervals of four days, of 1 loopful, 2 loopful, 0·2 c.c. 0·5 c.c., 1 c.c. and 1 c.c. respectively. The animals were later bled, but the result of agglutination tests with the two known strains, and the strains isolated in this investigation was confined to slight agglutination of the homologous strain at a titre of 1/10. This failure to produce agglutinins is in accord with Magnusson's findings (1940), but other workers have produced agglutinins readily (Bruner and Edwards, 1941 ; Merchant, 1935), and further work is thus being done on this point.

Since the agglutination tests were negative, precipitin tests were carried out according to the method described by Bruner and Edwards (1941), using the sera prepared against the isolated strains and against the known strains. The organisms from 24-hour agar slope cultures were suspended in saline, and 1/10th volume of N/2 HCl added ; the mixture was heated to 100° C. in a water-bath for 40 minutes, and then centrifuged. The supernatant fluid was neutralised with N/4 NaOH, centrifuged again, and the clear supernatant fluid decanted and used as antigen in the tests. Equal volumes of extract and test serum were employed. None of the sera prepared against the isolated strain by inoculating rabbits with killed organisms gave a positive result, but the sera prepared against the two known strains of *C. equi* by inoculating rabbits with living suspensions gave a positive result with extracts of the known and of the isolated strains. In all cases, a ring appeared at the junction of extract and serum immediately.

It may be concluded, in view of the positive precipitin tests obtained with sera from rabbits inoculated with known strains of *C. equi*, that the strains recovered from the tuberculous-like lesions and from the normal lymph node are probably *C. equi*. Owing to the difficulty already mentioned of obtaining agglutinating antisera, no conclusion can at present be reached as to whether the strains isolated are similar in respect of all their antigens.

PATHOGENICITY

A number of workers (*e.g.*, Magnusson, 1923 ; Bull, 1924 ; Dimock and Edwards, 1931 ; Rajagopalan, 1937) have recorded abscess formation in horses after subcutaneous inoculation of *C. equi*, and Magnusson reproduced the specific form of pneumonia after intratracheal administration. On the other hand, Dimock and Edwards (1931) obtained no result after intratracheal or oral administration. Plum (1940) injected suspensions of *C. equi* intravenously into swine without effect, while Magnusson produced local abscess formation in this species by subcutaneous inoculation. Feeding experiments in swine have produced varying results, for whereas Bendixen and Jepsen (1938) report localisation in the cervical lymph nodes, and nodular lesions in the lungs, Karlson and others (1940) found no gross or histological lesions at autopsy after giving large doses orally. A fibrino-purulent peritonitis, which was not necessarily fatal, was produced in g.-pigs after intraperitoneal inoculation by Bull (1924) and Magnusson (1938). In mice, subcutaneous inoculation was found by Miessner and Wetzel (1923) and Lütje (1923) to produce a local abscess, while Witte (1933) found that intraperitoneal or intravenous inoculation produced a hyperplastic spleen and necrotic foci in the spleen and liver. Magnusson (1938) and others found that rabbits are apparently not susceptible to administration by any of the usual routes.

The following is a brief record of the results of pathogenicity tests, using the strains isolated from pigs.

Rabbits.—1 c.c. of suspension, opacity 10, of a 24-hour agar plate culture was injected subcutaneously into one rabbit, and intravenously into another, without effect. The rabbit inoculated subcutaneously was normal when autopsied ten weeks later, while the rabbit inoculated intravenously was normal eight months later. However, the resistance of the rabbit does not seem to be absolute, as large doses (the whole of a 24-hour agar plate culture suspended in 5 c.c. saline) led to abscess formation in two other rabbits when inoculated subcutaneously ; the abscesses, which yielded *C. equi* in pure culture 24 hours after inoculation, healed in a few weeks, and the rabbits were otherwise unaffected.

Guinea-pigs.—Subcutaneous injection of a large dose (the whole of a 24-hour agar slope culture suspended in 1 c.c. saline) produced a local abscess in a few days, which reached its maximum size in a fortnight, occasionally (two out of nine cases) ulcerating through the skin. Healing soon followed, and, although the organism could be recovered, along with other bacteria (coliform organisms and streptococci) from the pus of a fully formed abscess, it was not demonstrated at autopsy after two to three months. Owing to the association of *C. equi* and tubercle bacilli in some of the original material from which the strains were isolated, it was thought possible that tubercle bacilli might be lying dormant in some of the cultures. For this reason, the guinea-pigs used were tested with avian and bovine tuberculin six weeks after receiving *C. equi* subcutaneously. None of the guinea-pigs reacted.

One guinea-pig died six days after receiving the whole of a 24-hour agar slope culture of one strain, suspended in 1 c.c. saline, intraperitoneally. Autopsy revealed a serofibrinous peritonitis with large fibrin deposits on the free border of the liver. In smears from the exudate, from which *C. equi* was recovered in pure culture, the organisms were found in clumps, mostly in cells, and some retained the red stain in the Ziehl-Neelsen method, while many of them showed a distinct beading when stained by Gram's method.

One guinea-pig was given 0·5 c.c., and each of two others 1 c.c., of a saline suspension of a 24-hour agar slope culture of one strain (opacity 2) intravenously, without effect. The g.-pigs remained well after inoculation, and were normal at autopsy, three months later.

Mice.—Intravenous or subcutaneous inoculation of 0·5 c.c. of a suspension, opacity 2, of a 24-hour agar slope culture, was without effect, but one of three mice that received the same dose intraperitoneally died in seven days with a peritonitis, and the organism was recovered from the peritoneal exudate in pure culture ; 1 c.c. of a filtrate from a culture grown for three days in serum broth at 37° C. was injected intravenously into each of three mice without effect.

Swine.—It was decided to use large doses of a mixture of all nine strains of *C. equi* isolated, to determine whether, under favourable conditions, the organism was capable of producing lesions that might be mistaken for tuberculous processes. Agar slope cultures of the nine strains used were each suspended in saline ; approximately equal volumes of the suspensions were mixed, and the pooled material was then sown on agar in large flat trays. After incubating for three days at 37° C., the resulting good growth was washed off, and suspended in saline, to such a density that when diluted eight times it had an opacity of 10. Eight newly weaned pigs were housed in groups of four in two separate pens. The pigs in one pen, numbered 3219, 3222, 3224, and 3227, were given 400 c.c. of the heavy suspension in their morning feed on each of six successive days, so that during the feeding experiment each should have received a total of approximately 600 c.c. These pigs all ate well during the experiment, and nothing was left in the trough. The pigs in the second pen received the heavy suspension parenterally. Pig 3221 received 5 c.c. intravenously, pig 3226, 1 c.c. intravenously, pig 3225, 5 c.c. subcutaneously, and pig 3223, 1 c.c. subcutaneously. All the test pigs received nicotinic acid during the first week of the experiment, and subsequently a supplement of dried yeast in their food, as a litter-mate had died at the start of the experiment with lesions suggesting a vitamin B deficiency. Within a fortnight of subcutaneous inoculation, pigs 3223 and 3225 showed discharging abscesses at the site of injection, but the regional lymph nodes were not palpably enlarged or painful. *C. equi* was recovered from the pus, along with streptococci and coliform organisms. For reasons already mentioned, the six pigs that were alive six weeks after the beginning of

the experiment were tested with avian and bovine tuberculin, but none reacted. The pigs were slaughtered after varying intervals of time. These details are shown in Table II.

Details of post-mortem examination of experimentally inoculated pigs.

(i) *Pigs receiving* C. equi *per os*

Pig 3219.—Killed after 83 days. No macroscopic or microscopic lesions were seen. *C. equi* was not detected by smears or culture in the lungs, liver, kidneys, spleen, submaxillary, mesenteric and portal lymph nodes, and tonsils.

Pig 3222.—Killed after 69 days. *C. equi* was recovered from the tonsils and lungs, but no macroscopic or microscopic lesions were found here or elsewhere in the body. *C. equi* was not recovered from the submaxillary, mediastinal, mesenteric and cervical lymph nodes, liver, spleen, and kidneys.

Pig 3224.—Killed after 76 days. No macroscopic or microscopic lesions were found. *C. equi* was not recovered from the liver, lungs, kidneys, spleen, submaxillary, cervical, mediastinal, mesenteric and caecal lymph nodes.

Table II

Pathogenicity Tests of *C. equi* in Pigs

Number of Pig	*Route of Administration of C. equi*	*Dose (c.c.)*	*Interval in Days to Killing*	*Result*
3219	Per os	600 approx.	83	No lesion
3222	„	„	69	„
3224	„	„	76	„
3227	„	„	48	„
3221	Intravenous	5	36	„
3226	„	1	96	„
3225	Subcutaneous	5	24	Local abscess
3223	„	1	90	„

Pig 3227.—Killed after 48 days. No macroscopic or microscopic lesions were found. *C. equi* was not recovered from the tonsils, submaxillary, mediastinal, cervical, and mesenteric lymph nodes, the liver, lungs, kidneys and spleen.

(ii) *Pigs receiving* C. equi *intravenously*

Pig 3221.—Killed after 36 days. No macroscopic or microscopic lesions were found in the lymph nodes or organs of the body, but *C. equi* was recovered from the lungs and mediastinal glands, spleen, kidneys, and the caecal and mesenteric lymph nodes. This finding suggests that once the organism has entered the body, it may persist for some time without producing any observable tissue changes.

Pig 3226.—Killed after 96 days. No lesions were detected macroscopically or microscopically. *C. equi* was not recovered from the lungs, liver, kidneys, spleen, tonsils, submaxillary, mediastinal, mesenteric, iliac and caecal lymph nodes.

(iii) *Pigs receiving* C. equi *subcutaneously*

Pig 3225.—Killed after 24 days. The local abscess was still discharging, but no other lesions were detected in the body. *C. equi* was recovered from

the local lesion, but not in pure culture, being associated with streptococci and coliform organisms. It was recovered in pure culture from the regional iliac and precrural lymph nodes, but these showed no lesions when examined microscopically. Cultures did not appear until eight days after inoculation of the media.

Pig 3223.—Killed after 90 days. The local abscess had healed, and was hardly detectable. No lesions were found, and *C. equi* was not recovered from the liver, lungs, kidneys, spleen, intestinal mucosa, caecal lymph nodes and follicles, submaxillary, mediastinal, mesenteric, precrural, popliteal, iliac and portal lymph nodes, and tonsils.

The serum from each of the eight pigs was tested for precipitins, but none reacted when added undiluted to pooled acid extracts of the strains used for inoculation.

These experiments in pigs indicate that *C. equi* has some pyogenic power, but when administered orally, subcutaneously or intravenously, in the manner indicated, it is not capable of setting up pathological processes resembling those of tuberculosis. When the organism has gained entrance to the body, it is capable of living in the tissues for some time without producing any lesions, and it is possible that it may act as a pyogenic secondary invader, as, for example, in contributing to the softening seen in tuberculous lesions.

DISCUSSION

Corynebacterium equi has been isolated from eight of 86 pigs, which showed lesions in the submaxillary lymph nodes resembling those of tuberculosis, and from one of 50 pigs with apparently normal glands. In five of the cases in the first group it was found to be associated with tubercle bacilli. A higher proportion of pigs has been shown to be infected in some countries: for example, Plum (1940) found that 60 per cent. of the swine carcases at one packing station that were thought to be affected with tuberculosis were in fact affected with *C. equi*. In another series of 1,280 cases, he found tubercle bacilli in 557, *C. equi* in 332, and neither in 391. Feldman and others (1940) examined a submaxillary lymph node, thought to show tuberculous lesions, from each of 89 swine carcases, and found *C. equi* in 20: seven of the strains were isolated from tissues apparently free from tubercle bacilli, although microscopical evidence of tuberculosis was found in two of these cases. They also found *C. equi* in the macroscopically normal lymph nodes of six out of 25 swine. Verge and Senthille (1942) recovered *C. equi* from the diseased lymph nodes of four out of 16 swine examined.

A study of the results obtained here and by other workers therefore leads to the conclusion that *C. equi* can be isolated from the normal cervical lymph nodes of pigs, and from a varying percentage of tuberculous-like lesions. The evidence obtained from the experiments reported here is in favour of the view that *C. equi* is not associated aetiologically with such tuberculous-like lesions.

SUMMARY

1. *C. equi* was isolated in eight cases from the supposedly tuberculous submaxillary lymph nodes of 86 pigs, being associated in five cases with tubercle bacilli.

2. From a series of apparently normal submaxillary lymph nodes from 50 pigs, *C. equi* was recovered in one instance.

3. Oral and intravenous administration of living suspensions of *C. equi* produced no observable lesions in swine. Subcutaneous inoculation led to local abscess formation.

4. Evidence is presented indicating that *C. equi* probably has no aetiological relationship to tuberculous-like lesions in the submaxillary lymph-nodes of swine.

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TUBERCLE BACILLI IN LESIONS OF THE SUBMAXILLARY LYMPH NODES OF SWINE

By

E. Cotchin

Research Institute in Animal Pathology, Royal Veterinary College, London

INTRODUCTION

In an investigation of the occurrence of *Corynebacterium equi* in the submaxillary lymph nodes of swine in this country recorded in this issue (Cotchin, 1943), a number of strains of tubercle bacilli were isolated; a brief account of these is given here. It is generally accepted from the results obtained by earlier workers that about two-thirds of the cases of swine tuberculosis in this country are caused by the bovine type of tubercle bacillus, and one-third by the avian type. With this knowledge available, it was felt unnecessary at the present time to study each strain in detail, but a limited number of cultural and pathogenicity tests made on the strains have given an indication of their probable types, and the classification of the strains is made with the proviso that it is not based on complete evidence.

MATERIAL EXAMINED

The submaxillary lymph nodes, showing what were apparently tuberculous changes, were taken from 86 pigs. As described in the previous paper, the lymph nodes were dropped into boiling water for several seconds, and then ground up, the suspension being treated with oxalic acid. Primary cultures were made on plain egg medium from the first 36 pigs examined, and cultures of tubercle bacilli were obtained from 28. In the case of the next 50 pigs, primary cultures were made as before, and guinea-pigs were also injected with material: tubercle bacilli were recovered from 40 of these pigs, in four cases by direct culture only, in 13 cases from the inoculated guinea-pigs only, and in 23 cases by both methods. The total number of pigs yielding tubercle bacilli was therefore 68. The strains from each of these pigs were cultured on plain egg and on 5 per cent. glycerin egg, and on the basis of their growth could be separated into two broad groups, A and B, with the cultural characters of the avian and the mammalian types respectively. Thus, group A contained those strains which grew well on plain egg, and showed improved growth on glycerin egg, while group B comprised those strains which grew poorly on plain egg, with no improvement on glycerin egg. The distribution of the strains in these groups is shown in Table I, which also shows how the strains were recovered by the different methods.

TABLE I
RECOVERY OF TUBERCLE BACILLI FROM LESIONS IN THE SUBMAXILLARY LYMPH NODES OF 86 SWINE

Tubercle Bacilli Recovered	*Nodes Examined by Culture only*	*Nodes Examined by Culture and Guinea-pig Inoculation*			*Total*
		Culture only	*Guinea-pig only*	*Culture and Guinea-pig*	
Group A	12	3	5	2	22
Group B	16	1	8	21	46
Negative	8		10		18
Total	36		50		86

CULTURAL CHARACTERS OF THE STRAINS OF TUBERCLE ISOLATED

Group A. Avian Type

Twenty-two strains fell into group A; the primary growths of these on plain egg medium showed individual colonies which were large, opaque, creamy, and easily emulsified. Growth was apparent in most cases within three to four weeks of inoculation. On glycerin egg, the confluent growth obtained was often heaped up, resembling worm casts, the colour developing from cream to brownish-yellow.

Group B. Mammalian Type

Forty-six strains fell into group B; their primary growths on plain egg showed either small rounded colonies, or a thin film of growth, often resembling frosted glass. The growth was tenacious and difficult to emulsify. Growth was apparent in most cases within four to six weeks of inoculation. As mentioned before, the presence of glycerin in the medium did not encourage growth.

PATHOGENICITY

Group A.—Suspensions of the 22 strains in this group were inoculated subcutaneously into guinea-pigs in doses of 1 mg. None of these guinea-pigs, when examined after periods of 59 to 142 days, showed lesions of progressive tuberculosis, although in 15 cases there was a small local lesion with caseo-pus, from which the strain was recovered. In no case were macroscopic lesions seen in the para-iliac or para-aortic lymph nodes, spleen, or liver.

Cultures of 12 strains of this group produced progressive tuberculosis in young cockerels in from 30 to 61 days (average 48 days) when inoculated subcutaneously in doses of 0·1 or 1 mg.; in some cases the disease was fatal, in others the cockerels were killed when showing great loss of condition. Miliary lesions were found in the

spleen and liver of all the inoculated birds. Acid-fast organisms were demonstrated microscopically and were recovered in cultures from the lesions. The alimentary tract appeared to be normal.

There is thus definite evidence that 12 of the 22 strains in group A are of the avian type. The other ten strains, from their cultural characters and their incapability of producing progressive tuberculosis in guinea-pigs, are probably of the avian type.

Group B.—(i) Sixteen of the 46 strains in group B were recovered from the submaxillary lymph nodes of 36 swine examined only by cultural methods. These strains were inoculated subcutaneously into guinea-pigs in doses of 1 mg., and all produced lesions of progressive tuberculosis, with a lesion at the site of inoculation, in the local gland, the para-iliac and para-aortic lymph nodes, and typical caseo-necrotic lesions in the liver and spleen, and usually with lesions in the lungs. The guinea-pigs were examined at intervals of 21 to 63 days after inoculation, with an average of 35 days.

(ii) One strain was isolated by direct culture only from one of the 50 pigs examined by cultural and guinea-pig inoculation methods ; the two guinea-pigs used unfortunately died in a few days, before lesions had time to develop. The primary culture was, therefore, inoculated subcutaneously into a guinea-pig in a dose of 1 mg., and produced a fatal progressive tuberculosis in 40 days.

(iii) Twenty-one strains of group B were isolated by direct culture, and also from the inoculated guinea-pigs. The latter showed in all cases lesions of progressive tuberculosis in periods ranging from 24 to 87 days, with an average of 37 days.

(iv) Eight strains of group B were recovered from inoculated guinea-pigs and not from direct cultures. In all cases, the guinea-pigs showed lesions of progressive tuberculosis, dying in periods ranging from 28 to 83 days, with an average of 40 days. In three cases, *C. equi* appeared in the primary cultures.

The 46 strains of group B, in virtue of the lesions present in the guinea-pigs inoculated with the original tuberculous material, or with cultures, are considered to be of the mammalian type. The poor growth on egg and glycerin egg points to the probability of their being of the bovine type.

DISCUSSION

From the 86 pigs examined, 46 strains of dysgonic mammalian type tubercle bacilli, and 22 strains probably of the avian type, were recovered ; five of the latter would not have been isolated unless guinea-pigs had been used.

Tubercle bacilli of all three types are capable of infecting swine, as shown by a table given by Cornell and Griffith (1930) ; they record that of 258 cases, the bovine type was found in 178, the human type in 5, and the avian type in 70 ; in four cases, both bovine and

avian types were found, and in one case, both human and bovine types. The proportion of bovine to avian strains is about 2·5 to 1, and the results here reported are in general agreement with this. However, the taking of individual lymph nodes from pigs at a bacon factory may give a false impression of the general incidence of the various types in swine, for there is a tendency for infections due to the avian type to be less severe, so that the apparent proportion of avian infections might be higher under these conditions ; further, the type of pig going to a bacon factory is not a true random sample of the whole pig population.

It has been suggested that the incidence of the various types of tubercle bacilli found in tuberculous swine in any country may parallel the incidence of tuberculosis in cattle and poultry in that country. This is likely to be near the truth in respect of those areas where swine are fed with uncooked material, or kept under free range conditions. It is known that most cases of porcine tuberculosis in the United States, where the incidence of bovine tuberculosis is very low, are due to the avian type (Schalk, 1931). Plum (1935) reports that on the island of Bornholm, which had been free from tuberculosis in cattle for more than a year, all of 100 porcine cases were due to the avian type. The findings of most workers suggest that infection spreads readily from cattle to swine, but the tendency for localization, and the early age at which most swine are slaughtered, reduces the spread from pig to pig, although such a method of spread cannot be ignored (McCarter and others, 1935). The majority of cases of bovine infection in swine in this country are due to infected cows' milk and of avian infection to contact with infected poultry, but the occurrence of tuberculosis in wild birds indicates another method of infection that may be of importance.

The failure to demonstrate tubercle bacilli in 18 of the 86 pigs (21 per cent.) is not inconsistent with the findings of others, even though animal inoculation was not used in the diagnosis of all our cases. For example, Cornell and Griffith (1930) failed in 7·8 per cent. of their cases, Graham and Tunnicliff (1926) in 27·1 per cent., and Feldman (1939) in 37·3 per cent. The reasons for this failure are not well understood ; it has been suggested that some of the lesions are not due to infection with tubercle bacilli, but are caused by *Corynebacterium equi* ; this view, however, is not generally accepted, and is not supported by the results of the writer's own investigation.

SUMMARY

1. From the submaxillary lymph nodes of 86 pigs, strains of tubercle bacilli were isolated in 68 cases.

2. Of these strains, 12 were of the avian type and a further 10 probably of the avian type, while 46 were of the mammalian, and probably bovine, type.

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BRILLIANT GREEN—NEUTRAL RED—LACTOSE AGAR FOR THE ISOLATION OF *S. SUIPESTIFER* FROM PIG FAECES

By

GEORGE SLAVIN,

Veterinary Laboratory, Ministry of Agriculture and Fisheries, New Haw, Weybridge

INTRODUCTION

IN work at this laboratory on pig paratyphoid the isolation of *Salmonella suipestifer* from faeces and other heavily contaminated material using a number of direct plate media and enrichment methods proved unsatisfactory. The media comprised MacConkey plates, Endo plates, brilliant green peptone water, brilliant green broth, buffered brilliant green peptone water, and Müller's original tetrathionate broth. This last medium was tested more extensively than any other.

Although Kauffmann's tetrathionate-brilliant green-bile enrichment medium is probably the most effective yet devised, it was not used because of the difficulty of obtaining the dye specified by him. The organisms normally present in pig faeces rapidly rendered inert the inhibiting substances in the enrichment media and grew so prolifically on the solid media that any salmonella colonies present were obscured. A solid medium containing brilliant green is little used in this country or America although it is extensively used on the Continent as a standard medium, mainly in conjunction with enrichment methods (Kauffmann, 1935 and 1941). In the latter part of 1940 it was officially prescribed for bacteriological meat inspection in Germany (Köser and Wittenberg, 1941). Perry and Bensted (1929) apparently reflect general opinion in this country, stating " In the original application of this observation [the selective action of brilliant green] the dye was incorporated in a solid medium, but it was not until it was realised that the use of these dyes was of little practical value except in a fluid medium that their employment in the isolation of the enteric group of organisms became general."

Teague and Clurman (1916) and Holt-Harris and Teague (1916) published extensive work noting the variable action of the dye and stressing the need to titrate it with each culture medium prepared. This variable action explains why the dye has been so little used in solid media: that it is any less variable in liquid media has not, however, been demonstrated. Teague found that the action of brilliant green was improved by combining it with eosin or similar dyes. Jones (1936) reporting on brilliant green-eosin agar found that different samples of eosin varied but the two samples of brilliant green tested did not differ appreciably. Many authors, when prescribing the mode of preparing brilliant green media, merely stipulate a certain concentration of brilliant green and ignore the variation in inhibitory action of different samples of the dye. Despite the importance of the subject and the usefulness of brilliant green, dye manufacturers have not issued it in standard strength, and, as has been proved by several workers, samples vary.

PRELIMINARY OBSERVATIONS

For the work on hand, brilliant green agar seemed to be the most promising medium, mainly because it is easily prepared and keeps well. As a first step, the brilliant green was titrated in plain nutrient agar against our No. 1 strain of *S. suipestifer* to find the largest amount of the dye which would allow growth. It was noted that 48 hours had to be allowed to obtain full growth but this was no impediment to this work. The most suitable dilution was 1/10,000 for the available sample of dye. The medium was then made a differentiating one by incorporating 1/10,000 neutral red and 1 per cent. lactose. Plates inoculated with a 7 mm. loopful of a 25 per cent. suspension of normal pig faeces containing known numbers of *S. suipestifer* showed little or no growth of contaminants and a heavy growth of *S. suipestifer*.

A further test was made of the effect of this medium on contaminants by plating numerous samples of faeces from laboratory pigs. No salmonella colonies were found and in most cases the plates were nearly clear of contaminants. An extensive growth of Proteus appeared on a few plates.

So far only one strain of *S. suipestifer* had been tested, and it was necessary to discover if other *suipestifer* and salmonella strains were equally resistant. Twenty-six salmonella strains, mostly *suipestifer*, were therefore sown on plates using a small loopful of young broth culture. All grew well with the exception of one strain, which was completely inhibited, and another strain which grew sparsely. This latter strain, however, grew fairly well at room temperature during the few days it was left on the bench after incubation.

PREPARATION AND USE OF BRILLIANT GREEN AGAR

Brilliant green in solution is added to plain nutrient agar, pH 7·5, to make a suitable concentration for the laboratory medium in use. With one sample of dye the concentration found most suitable here was 1/10,000. Dye from another source was found to be about twice as inhibitory. Duplicate tests with the two samples gave strikingly different results, and it was found necessary to reduce the concentration with the second sample to 1/20,000. Depending upon the dye used a second plate of half the usual strength may be included; it is likely, however, that there are sensitive strains which are inhibited even by very high dilutions of brilliant green. The medium is made a differentiating one by adding 1/10,000 neutral red and 1/100 lactose. Every batch of medium does not need standardisation because nutrient agar made with the same materials is of fairly constant composition.

When the medium is first used it is advisable to inoculate plates with faeces known to contain salmonella organisms so as to become accustomed to the appearance of the colonies on the medium. The medium dried ready for use is brown with a tinge of violet. Remarkable colour changes are produced by the growth of contaminants —light green, red, violet, blue and bright yellow. A yellow colour,

sometimes in the case of salmonellas accompanied by the formation of reddish brown crystals in the depth of the medium, is characteristic of a heavy growth of salmonellas, Proteus, and a few other organisms. Lactose fermenters grow as red colonies and sometimes colour the adjacent medium red, but the red rarely extends far over the plate. Late lactose fermenters present are always a perplexing problem but the colonies are usually distinguishable from salmonella colonies by their appearance. As a rule, contaminants are few in number but for some unknown reason there may be a heavy growth of them, though it is rarely so heavy as to obscure salmonella colonies. However, the characteristic colour changes produced by salmonellas may be obscured, especially when there are few of the latter, and the colonies have then to be picked on their general appearance. When contaminants are entirely absent it has been remarked that widely spaced, small salmonella colonies do not change the colour of the medium, and these colonies are themselves stained a lilac colour. Occasional salmonella colonies have a faint red centre at the surface of the medium which is due to the fermentation of substances other than lactose in the medium. Dwarf colonies, usually accompanied by normal colonies, are occasionally seen, especially with sensitive strains.

The principal contaminants on the plates are Proteus and *Ps. pyocyanea*. Proteus sometimes completely smothers all other growth, probably as a result of insufficient drying of the medium. *Ps. pyocyanea* grows fairly well but is restricted in area. No difficulty has been experienced with agglutination tests of salmonellas grown on this medium. Colonies were tested by the usual spot test technique and all, including even the dwarf colonies, reacted normally.

An extensive test of the medium described above was then made, comparing it with a number of other selective media, the method being to test all stock strains of salmonella by adding known numbers to normal pig faeces.

METHOD

To each of five small beakers (50 c.c.) containing 20 c.c. of saline, sufficient broth culture was added to make dilutions of 1/2,000,000, 1/1,000,000, 1/500,000, 1/250,000 and 1/125,000 of the broth culture. Five grams of faeces were added to each beaker and well mixed, using a separate glass rod for each beaker. After standing a few minutes to allow gross particles to settle, 7 mm. loopfuls were inoculated on the appropriate plates and into enrichment broth. The broth culture was counted in the usual way with three poured plates. The 7 mm. loop was made of stout nichrome wire and lifted a comparatively large volume of fluid. In the first test each loopful was weighed by subtraction and about 100 per cent. variation in the volume taken up was found. An average of 0·062 c.c. fluid was used in this way in estimating the number of salmonellas

inoculated on to the plates. Throughout the series of tests suspensions of faeces containing salmonellas were sown on brilliant green agar plates and on brilliant green-telluric acid enrichment broth, the latter being plated after 24 hours and 48 hours incubation on litmus lactose agar and brilliant green agar plates. Later, Endo and MacConkey plates were also used. Small modifications were introduced as the tests proceeded. At first a 1/1,000,000 dilution of culture in a 25 per cent. suspension of sterilised faeces was plated on plain agar, but as this was merely a doubtful check on the poured plates it was abandoned. Instead a 1/2,000,000 dilution of culture in saline was plated on Endo, MacConkey and brilliant green plates and also inoculated into brilliant green-telluric acid enrichment broth. In Table II are summarised the results of tests with 28 strains using the method finally adopted.

RESULTS

Table I shows the detailed results with four salmonellas. The numbers shown in the salmonella column are those of colonies spaced well apart and apparently the growth of single organisms on the plate. " Numerous " means either that the colonies were in isolated groups and that it was unsafe to assume that each colony represented a single organism in the inoculum or that the plates were so crowded that it was impossible to count the colonies accurately. This growth of colonies in groups could have arisen either from insufficient drying of the agar with resulting tendency for droplets of expressed water to form on the surface during the early stages of incubation or from excessive drying of the agar resulting in the inoculum becoming so rapidly soaked up that it would not spread evenly. The line of figures marked " control " shows the number of colonies on plates inoculated with a 7 mm. loopful of a 1/2,000,000 dilution of broth culture in saline. The results with the four strains of *suipestifer* are selected as typical of those to be expected with this medium. The first two, *S. suipestifer* (Budapest D) and *S. suipestifer* (Lewis), are representative of the majority of the strains that have undergone test. The two strains grew well on the medium with a complete absence of contaminants in the case of the first strain, and numerous contaminants on the 1/20,000 plate sown with the two highest dilutions in the case of the second strain. These contaminants apparently suppressed the growth of the salmonellas because there were no salmonella colonies on the first plate and only seven on the second. The third plate, with only a few contaminants, agreed well with the 1/10,000 plate. *S. suipestifer* (J.171) is a brilliant-green sensitive strain and failed entirely to grow on this medium. It also failed to grow on the control MacConkey plate inoculated with a 1/2,000,000 suspension of culture in saline without faeces. The numbers of salmonella colonies on the plates on which growth occurred showed an unexpectedly close

agreement with the probable number of salmonellas in the inoculum. The outstanding exception was the control inoculation with *S. suipestifer* (Lewis) on Endo medium. As already stated, the volume lifted by the 7 mm. loop was found to vary by about 100 per cent.

The MacConkey and Endo plates, with only two exceptions, showed in each case such a heavy growth of contaminants as to obscure single colonies. This heavy growth was to be expected in view of the volume of the inoculum but reduction in the volume of the inoculum would have decreased correspondingly the chances of recovering salmonellas in the higher dilutions. The tests of the brilliant green-telluric acid broth show up rather better in this table than in the general series of tests. In most tubes there was a heavy growth with a black deposit on the bottom.

Table II shows the results of tests upon 28 salmonellas, mostly *S. suipestifer*. In the table, plates are shown as positive when any salmonella growth developed irrespective of the number of salmonella colonies on the plate. It will be seen that in a few instances the 1/10,000 brilliant green plates were positive but the 1/20,000 were negative and the converse occurred but still more rarely. The reason for this was that the number of colonies which developed on the plates were few in number and it was probably a matter of chance on which plate they developed. Summarised, Table II shows that 23 of the 28 strains (which included four strains of salmonella other than *suipestifer*) were recovered on the brilliant green plates whereas on Endo and MacConkey plates only two strains were recovered. With the use of brilliant green-telluric acid broth one strain only was recovered and then only by adopting a modification of the method, *viz.*, inoculation on to brilliant green agar. Including the tests shown in Table II, this broth was inoculated 165 times with faeces known to contain salmonellas and the only positive result was that just mentioned with one strain only. In addition, it was inoculated 22 times with a 1/2,000,000 suspension of salmonella in saline without faeces and failed invariably to yield growth.

DISCUSSION

Brilliant green agar, with the addition of neutral red and lactose, was greatly superior to Endo and MacConkey plates and to brilliant green-telluric acid enrichment broth for recovering salmonellas from artificially infected pig faeces. In spite of the high efficacy of the brilliant green plate, it must be allowed that a direct method is inferior to a good enrichment method because in naturally infected faeces salmonellas are probably present in most cases in small numbers. In such circumstances it is impracticable, even with the most satisfactory plate medium, to inoculate enough faeces to be sure of obtaining a positive result. It is then largely chance which decides whether or not a particular sample of faeces containing salmonellas is positive on culture. When chance plays so large a part it is of

first importance to select a method which on test gives the greatest hope of success. Tradition rather than efficacy is probably responsible for the continued use of many media which repeated tests have proved to be inefficient.

SUMMARY

By direct plating on brilliant green-neutral red-lactose agar, 23 out of 28 salmonella strains, mostly *suipestifer*, were recovered from artificially infected pig faeces. By plating on Endo and MacConkey agar two strains only were recovered. By inoculation of brilliant green-telluric acid enrichment broth which was plated at 24 and 48 hours on litmus lactose agar not one strain was recovered.

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TABLE I

DETAILS OF TESTS WITH FOUR STRAINS OF SALMONELLA

Organism	Approx. number Sal. inoculated calculated from plate counts	*Brilliant Green Agar*				*Endo*		*MacConkey*		*Brilliant Green-Telluric Acid Broth*							
										24 *Hours*				48 *Hours*			
		1/10,000		1/20,000						*Litmus Lactose Agar*		*Brilliant Green Agar*		*Litmus Lactose Agar*		*Brilliant Green Agar*	
		Contam	Sal	Contam	Sal	Contam	Sal	Contam	Sal	Contam	Sal	Contam	Sal	Contam	Sal	Contam	Sal
S. suipestifer Budapest D	15	0	8	0	10	+++	0	+++	0	0	0	0	0	0	0	0	0
	30	0	16	0	24	+++	0	+++	0	0	0	0	0	0	0	0	0
	60	0	30	0	36	+++	0	+++	0	0	0	0	0	++	0	0	0
	120	0	Num	0	Num	+++	0	+++	0	0	0	0	0	0	0	0	0
	240	0	Num	0	Num	+++	0	+++	0	0	0	0	0	0	0	0	0
Control	15	0	14	0	12	0	15	0	10	0	0	0	0	0	0	0	0
S. suipestifer Lewis	27	0	21	++	0	+++	0	+++	0	+	0	0	0	+	0	0	0
	54	0	48	++	7	+++	0	+++	0	+	0	0	0	+	0	0	0
	108	0	93	+	88	+++	0	+++	0	+	0	0	0	0	0	0	0
	216	0	181	0	222	++	Num	++	Num	+	0	0	0	++	0	0	0
	432	0	Num	0	Num	++	Num	++	Num	+	0	0	0	+++	0	0	0
Control	27	0	29	0	33	0	54	0	26	0	0	0	0	0	0	0	0
S. suipestifer J.171	22	0	0	0	0	+++	0	+++	0	+	0	0	0	++	0	0	0
	44	0	0	0	0	+++	0	+++	0	++	0	0	0	++	0	0	0
	88	0	0	+	0	+++	0	+++	0	++	0	0	0	++	0	0	0
	176	0	0	0	0	+++	0	+++	0	++	0	0	0	++	0	0	0
	352	+	0	0	0	+++	0	+++	0	++	0	0	0	++	0	0	0
Control	22	0	0	0	0	0	30	0	0	0	0	0	0	0	0	0	0
S. enteritidis	30	+	0	+	0	+++	0	+++	0	+	0	0	0	+	0	0	0
	60	+	0	+	0	+++	0	+++	0	0	0	0	0	0	0	0	0
	120	+	0	+	0	+++	0	+++	0	0	0	0	0	0	0	0	0
	240	+	0	+	0	+++	0	+++	0	+	0	0	0	0	0	0	0
	480	+	0	+	0	+++	0	+++	0	+	0	0	0	+	0	0	0
Control	30	0	0	0	0	0	Num	0	24	0	0	0	0	0	0	0	0

Contam = Contaminants, organisms other than salmonella.
Sal = Salmonella.
\+ = Small number of contaminants.
++ = Numerous contaminants but colonies well apart.
+++ = Heavy growth of contaminants but still possible to pick individual colonies.
0 = No growth.
Num = Numerous colonies.

TABLE II

Organism	*MacConkey*	*Endo*	*Brilliant Green Agar*		*Brilliant Green–Telluric Acid Enrichment Broth*	
			1/10,000	1/20,000	24 *Hours*	48 *Hours*
	Pos.	*Pos.*	*Pos.*	*Pos.*	*Pos.*	*Pos.*
S. suipestifer Findlay	0	0	5	5	0*	0*
S. suipestifer Hale	0	0	5	4	0	0
S. suipestifer Lewis	2	2	5	4	0	0
S. suipestifer Day	4	4	5	5	0	0
S. suipestifer Craig	0	0	5	0	0	0
S. suipestifer Enfield	0	0	5	0	0	0
S. suipestifer Gee	0	0	5	5	0	0
S suipestifer L.C.S.	0	0	5	5	0	0
S. suipestifer St. James	0	0	3	2	0	0
S. suipestifer Cole	0	0	5	5	0	0
S. suipestifer Budapest D	0	0	5	5	0	0
S. suipestifer Dudgeon	0	0	5	5	0	0
S. suipestifer Johns Hopkins	0	0	5	5	0	0
S. suipestifer S.F. 8	0	0	5	5	0	0
S. suipestifer J.171	0	0	0	0	0	0
S. suipestifer S.F.24	0	0	5	5	0	0
S. suipestifer M.H.	0	0	5	5	0	0
S. cholerae suis	0	0	1	1	0	0
S. suipestifer J.174	0	0	0	0	0	0
S. suipestifer Hog cholera	0	0	2	2	0	0
S. suipestifer Indiana 49	0	0	5	5	0	0
S. suipestifer Plymouth	0	0	0	0	0	0
S. suipestifer Budapest B	0	0	5	5	0	0
S. suipestifer L.S.A.	0	0	5	5	0	0
S. paratyphi A	0	0	3	4	0	0
S. enteritidis	0	0	0	0	0	0
S. paratyphi B	0	0	5	5	0	0
S. typhi murium	0	0	0	0	0	0

* = Pos. on brilliant green plates inoculated from the broth.

Summarised results of tests of 28 strains of salmonella with the technique described. In each test five different dilutions, as in Table I (see text) of culture in faeces were plated and the figures under Pos. show the number of dilutions from which salmonella colonies developed. Numbers less than five in the Pos. column indicate that the organism was sensitive to brilliant green and grew only from the inocula containing the larger numbers of salmonellas.

HISTIOCYTIC AND FIBROPLASTIC SARCOMA (MIXED-CELL SARCOMA) IN THE DOMESTIC FOWL

By

J. G. Campbell,

The Department of Poultry Diseases, Royal (Dick) Veterinary College, Edinburgh

INTRODUCTION

A number of spontaneous connective tissue tumours occur, particularly in birds, the assessment of which offer considerable difficulty, owing to the apparent lack of a type cell, and the extraordinary pleomorphism of the cellular elements composing them. Such tumours are well known in human pathology, but there is very little discussion of them in standard textbooks, and most authorities avoid assigning any cell type to them.

MacCallum (1937) describes tumours which are characterised by cells showing great variation in shape and size, some of them containing very large cells (tumour giant cells) showing much diversity of form, and bizarre modifications of their nuclei. Such tumours he calls " mixed-cell sarcomas."

Sarcomata are common in the fowl, though whether the inclusion under this heading of such conditions as the leucoses is desirable is open to question. This is at any rate the practice of Malke (1930) who, in his paper on " Neoplasms in the domestic fowl," classifies 66 per cent. of his sarcomata as " sarcoma mixtocellulare "—including lympho-sarcomata or aleukaemic lymphoid leucosis.

Jackson (1936) has suggested a cytological classification of these mixed-cell tumours in conformity with the growing tendency to avoid a purely morphological nomenclature. Thus he arranges them under three general headings : (1) Fibroblastic (or better, fibroplastic, since it indicates the morphological multipotentiality of the cell) ; (2) mixed cell (*i.e.*, histiocytic and fibroplastic) sarcoma ; and (3) histiocytic sarcoma. He points out, however, that difficulty may arise over intergrades and this fact demonstrates that all types are but variants of one basic neoplastic cell type.

Carrell and Ebeling (1926) and Haddow (1933) have published work suggesting that the macrophage of the fowl can be changed into a neoplastic histiocyte or a Rous sarcoma cell. This view, however, is not in accordance with the findings of Ludford (1936) or Halberstaedter *et al* (1941), whose results appear to show that it is the fibroblast and not the macrophage which fixes and is cancerised by the virus of Rous or Fujinami tumours.

In certain strains of the Rous sarcoma, mixed-cell growths have appeared: conversely McGowan (1928) refers to naturally occurring mixed-cell tumours as being sarcomata of the Rous type.

Maximow and Bloom (1939) say that in inflammation, the fixed histiocyte or macrophage may be regarded as a modified fibroblast, differing from the fibroblast *per se* in that it tends to be stellate and to form reticulin instead of collagen fibrils. Besides fibroblasts, it can also produce free phagocytic cells such as monocytes and giant cells. Some workers claim it can produce the free multipotential precursors of the circulating blood elements (the haemocytoblasts of the Monophyletists), although Maximow denies this.

It will be seen then that mixed-cell tumours may well show all these forms of cellular elements ; and in those that have been sub-

jected to critical cytological examination, especially by Jackson, the collagen producing fibroblast, the reticulin producing fixed histiocyte, and the phagocytic free histiocyte or macrophage, together with groups of primitive blood cells have all been recognised.

A study of these new growths, therefore, is likely to throw fresh light on a number of debatable histogenetical problems, and the case described in detail below appears to confirm several debatable points. The tumours occurred in a Buff-rock hen, weight 4½ lb., which had been killed by dislocation of the neck.

MACROSCOPICAL EXAMINATION

Post-mortem examination showed both lungs to contain many firm, white tumours growing in the form of rounded masses. These protruded somewhat from the surface, giving each lung a nodular appearance. On section, these masses were seen to be present throughout the lung, and in places they were merged to form diffuse white areas. The average diameter of the isolated growths was 3 to 4 mm.

There was a firm whitish tumour growing in the anterior lobe of the right kidney (25 × 22 × 17 mm.). This was firmly attached to the 6th-7th ribs and their costo-vertebral junctions by connective tissue. There was no erosion of the ribs. The left kidney appeared normal. The right adrenal gland was somewhat larger than normal, measuring 12 mm. in cross-section as against the normal 7 to 8 mm. Its cut surface had a greyish appearance. The left gland was normal in size and colour.

The oviduct was impacted with inspissated yolk, and had several small (0·2 mm.) shining nodules growing in the wall of the magnum. The ovary, alimentary tract, liver, spleen, thyroids, blood and bone marrow all appeared normal.

Tissue taken from lung, kidney, adrenal and oviduct, as representing the abnormal organs, and from the above-mentioned macroscopically normal organs, was fixed in 10 per cent. formalin in physiological saline. Paraffin and frozen sections were cut. The following stains were used on the paraffin sections: Harris's haematoxylin and eosin, phosphotungstic acid haematoxylin, thionin blue, a modified Mallory stain and the Gros-Schultze method for nerve-fibres. Frozen sections were impregnated by both Cajal's silver and gold chloride-sublimate methods. Further formol fixed material was impregnated by Levaditi's method, embedded and paraffin sections cut at 15μ.

MICROSCOPICAL EXAMINATION

Lung.—A general low power survey of the lung shows the tumours to be growing in the walls of the alveoli. All stages of involvement are seen from a minute growth in one wall to complete obliteration of the normal lung structure over an area corresponding to 40 to 50 alveoli. The pulmonary vessels are all congested.

Examination under higher power shows a great diversity of cellular elements in different parts. These may be classified as follows: (1) fixed histiocytes, (2) free macrophages, (3) fibroblasts,

(4) tumour giant cells, and (5) lymphoid and myeloid elements. All degrees of transition exist between the first four types.

Owing to the inferior cytology of the lung tumours compared with that in other organs, a detailed description of the cells will be given later, and only a general account will be given of the lung. The histiocytic type of cell predominates in the lung tumours. These are characterised by great pleomorphism, but in the main they are elongated cells, with many fine protoplasmic prolongations which frequently merge with those of neighbouring cells. The histiocytes lie in loose interweaving strands and fasciculi (Fig. 1). In many areas the fixed histiocytes take on a more rounded shape, and judging from their amoeboid forms and the fact that they often contain cellular debris, they are actively motile and phagocytic.

Differentiation to the fibroblast type has taken place in other areas, and these cells are always associated with abundant collagen fibres. Groups of giant cells occur. They are frequently multinucleated and exhibit active phagocytosis. They may be lying in a reticular mesh, or occurring free in the lumina of the alveoli, together with a pink-staining homogeneous material.

The bronchial submucosa contains large numbers of plasma cells and a few lymphocytes. In certain parts a myeloid type of cell with eosinophilic granules in the cytoplasm predominates. Portions of bronchial epithelium in the form of a few palisaded columnar cells are distinguishable in parts of the tumour adjacent to the bronchi.

Reticulin fibrils are intimately associated with the fixed histiocytic moiety. They run mainly parallel to the long axis of the cell and branch frequently. The giant tumour cells usually lie enclosed in a reticulin mesh.

Kidney.—The growth in this organ has a similar structure to those in the lungs, with the exception that although the strands and bundles of fixed histiocytes still occur, they are not so much in evidence, the main part of the tumour being composed of neoplastic fibroblasts and groups of large roughly spherical cells lying in a loose framework of fixed histiocytes. The growth is not encapsulated, and is actively invading the kidney substance. A few delicate blood vessels occur. Some isolated sections of renal tubules remain in the invading mass.

High power shows the histiocytic element to be similar to that in the lung. The cells, which are very faintly staining, measure about $5 \times 17{\cdot}5\mu$, although it is difficult to arrive at an accurate measurement of the length owing to the tapering of the cytoplasm into fibrillar prolongations. The nuclei are pale, vesicular and oval and measure about $6{\cdot}25 \times 3{\cdot}75\mu$.

The free macrophages measure 7 to 14μ in diameter. They have a foamy, somewhat basophilic cytoplasm, a prominent vesicular nucleus with well-marked nuclear membrane, and a large acidophilic nucleolus. The giant phagocytic cells measure anything from

20 to 50μ in diameter. Generally speaking, the larger the cell the more frequently it is multinucleated. Cells showing amitosis are not uncommon. They frequently contain eosinophilic leucoctyes lying in vacuoles in their cytoplasm. These large cells lie in the meshes of a fibrillar plexus. With their basophilic granular cytoplasm, large vesicular nucleus and prominent nucleolus they bear a strong resemblance to unipolar ganglion cells (Fig. 2). Thionin blue demonstrates a certain amount of granular substance in the cytoplasm. The nuclei of some of these tumour giant cells are often very large and bizarre. They may be indented, twisted, bent into semi-circles and be anything up to 17μ in diameter. The nucleolus also varies greatly in size, one measuring 15μ across. There may be two nucleoli present. Numbers of these giant phagocytic cells lie free in the lumina of the large proximal collecting tubules.

The fibroblasts occur as fairly dense bundles of elongated cells measuring about 30 to 50μ $\times$ 3 to 4μ. Here again it is difficult to arrive at an accurate figure. They have a faintly basophilic cytoplasm which tapers to a fine thread at each pole and a large oval or round vesicular nucleus with a well-defined nuclear membrane and a few coarse chromatin granules connected by linin threads. There is a prominent acidophilic nucleolus ; sometimes there are two such nucleoli. The prolongations of these cells stain as for collagen with Mallory's connective tissue stain, and Anderson's modification of Jakob's stain for nervous tissue gives an excellent demonstration of the intimate association between cytoplasm and collagen production, the former staining red and the latter blue. (Fig. 3.)

In view of the resemblance to ganglion cells, several impregnation methods were used, but neither Cajal's silver method nor Levaditi's method demonstrated neurofibrils. Similarly an attempt was made to demonstrate neuroglia by Cajal's gold chloride-sublimate method, with negative results. A study of the recticulum shows a rich plexus of reticulin fibrils enmeshing the giant cells, and an abundance of delicate branching fibrils in intimate relation to the more undifferentiated (histiocytic) part of the tumour. Very few argyrophil fibres occur amongst the fibroblasts.

Adrenal Gland.—The capsule of the gland is intact. There is a group of normal ganglion cells attached to the outside of the capsule. The medulla has completely disappeared, its place being taken by small groups of round or polygonal cells, and strands and bundles of interweaving elongated fibroblasts. A few giant phagocytic cells are scattered throughout this tissue. Islands of cortical cells remain intact.* (Fig. 4.)

* In his discussion of secondary tumours in the adrenals, Willis (1934) points out that : " Despite the intimate anatomical relationship of the cortex and medulla, the cortex long resists neoplastic invasion from medullary deposits. No mechanical or vascular conditions suffice to explain this, and serious consideration must be given to the view that the metabolic qualities of the adrenal cortical tissue are such as to inhibit tumour invasion."

Transition forms between the macrophages and the giant phagocytic cells can be seen. Some of these intermediate types of cell show an indistinct or no nuclear membrane, although the nucleolus is still prominent. The cytoplasm is coarsely granular or hyaline. These are probably degeneration forms. Occasionally large " dumbbell " shaped forms occur. They have a rather coarse granular cytoplasm with a small basophilic nucleus at each pole. Such cells appear to be undergoing amitotic division.

The rest of the cellular elements are as already described in the lung and kidney. The intercellular substance is composed of fibres which stain very faintly with eosin, but show up better when Mallory's connective tissue stain or phosphotungstic acid haematoxylin is used. The fibres can then frequently be traced to cytoplasmic prolongations of the fibroblasts. A few delicate blood vessels traverse the tumour. Wandering cells of the myeloid series occur in the intercellular stroma.

Laidlaw's silver impregnation shows wavy reticulin fibrils branching throughout the tissue replacing the medulla. The fibrils enmesh small groups of cells and even individual cells of the large type. The cortical cell islands appear normal and are not pervaded by reticulum.

Other Organs.—The liver, spleen, ovary, adjacent sympathetic ganglia, and longitudinal sections of marrow in rib and femur show no abnormality.

The nodules growing in the wall of the magnum of the oviduct are fibro-adeno-leiomyomata.

DISCUSSION

The case is an unusual one as regards the primary site (kidney) and the distribution of metastases. In Jackson's (1936) ten recorded cases, the commonest primary site was the ovary (5), other sites mentioned being the wattle (1), oesophagus (1) and unrecorded (3). The commonest sites for metastatic growths were bowel serosa and mesentery (strictly speaking, peritoneal implantation) from the primary ovarian tumour. True haematogenous metastases were observed in liver, spleen, kidney, heart and muscle, each in one case respectively.

Another interesting point is the very close resemblance of some of the giant phagocytic cells to ganglion cells, and the coincidence of neoplasia in the adrenal medulla—a common site of origin for neuroblastomata and ganglion-neuromata. However, the large cells frequently show cyto-phagocytosis ; no neuroblasts are present, no neuro-fibrils can be demonstrated, the presence of reticulin fibrils (*vide* Foot and Day, 1925) and the normal appearance of the ganglion cells adjacent to the adrenal and ovary, all point to the growth not being a neurogenic one.

The direct transformation of cytoplasm to collagen—a process which has been the subject of much controversy—is well shown in

this tumour. Two schools of opinion exist regarding the formation of collagen, one holding that it is laid down between the cells through a condensation of liquid intercellular substance secreted by the cells, and the other that it originates by direct transformation of cytoplasmic prolongations.

The multipotentiality of the histiocyte is well illustrated in this case. All transition forms from this cell, whether fixed or mobile, through fibroblasts to multinucleated phagocytic giant cells exist. The resemblance of those parts of the tumour which consist of loosely arranged neoplastic histiocytes to the Rous Sarcoma No. 1, is very striking.

SUMMARY

A histiocytic and fibroplastic sarcoma (" mixed-cell sarcoma "), the primary growth probably being in the right kidney, with metastases to the right adrenal and to both lungs.

ACKNOWLEDGMENT

I wish to express my thanks to my colleague, Mr. P. G. D. Morris, of the Anatomy Department, Royal (Dick) Veterinary College, for the photomicrographs.

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LEGENDS TO PLATE I

FIG. 1. Portion of lung tumour, showing neoplastic tissue composed of fixed histiocytes. Mallory's connective tissue stain. × 200.

FIG. 2. Giant phagocytes (macrophages) in the kidney tumour. Note resemblance to ganglion cells. Thionin blue and eosin. × 350.

FIG. 3. Fibroblastic cells, also from the kidney tumour. The long collagen fibres arising from the cells are well shown. The intra-cytoplasmic formation of collagen can be seen in the two macrophages to the right of the photograph. Anderson's stain. × 500

FIG. 4. Neoplastic fibroblasts in the adrenal medulla. The cortex is not affected. Thionin blue and eosin. × 500.

PLATE I.

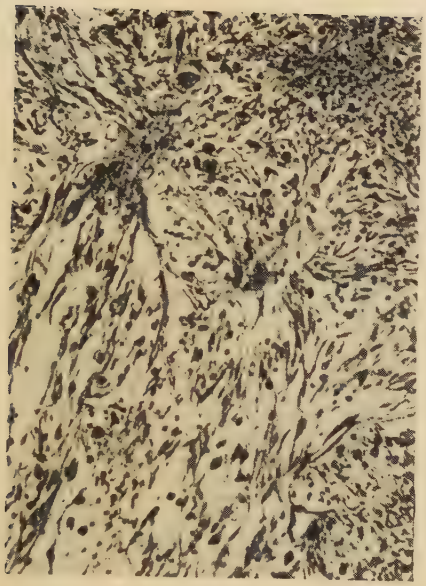


FIG. 1

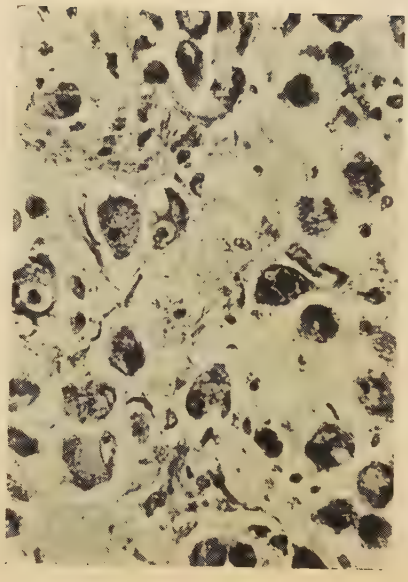


FIG. 2

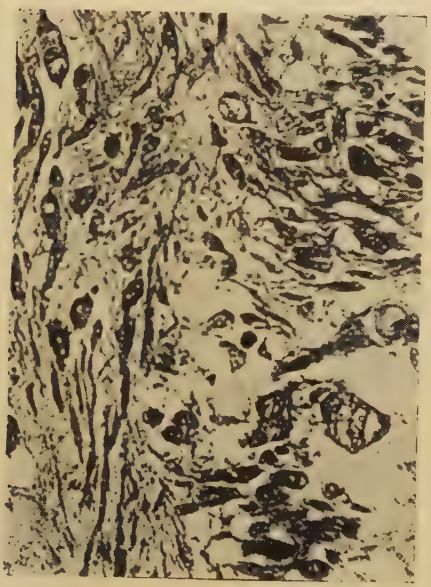


FIG. 3

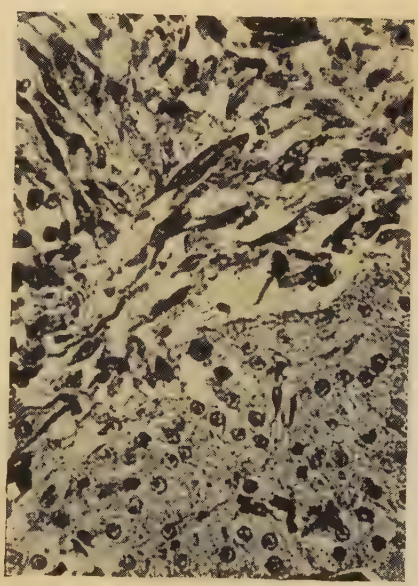


FIG. 4

THE ANTIGENIC STRUCTURE OF BRITISH STRAINS OF SWINE INFLUENZA VIRUS

By

R. E. Glover and C. H. Andrewes

Farm Laboratories, National Institute for Medical Research, Mill Hill, N.W.7

INTRODUCTION

A respiratory disease of pigs resembling clinically the swine influenza described by American workers has been recorded in the British Isles (Lamont, 1938; Blakemore and Gledhill, 1941). Filtrable viruses pathogenic for the ferret were isolated by the latter workers cited, from natural cases and also from pigs which had been infected artificially by intranasal instillation of suspensions of the lungs of sick animals. Lamont succeeded in infecting piglets experimentally with material derived from outbreaks in Scotland and N. Ireland. He pointed out that in these areas the disease was observed to be more destructive and to run a more chronic course than American swine influenza and seemed in these respects to resemble more closely the *ferkelgrippe* of German authors. The English workers have also encountered a form of the disease with a chronic phase and have recovered virus from cases of long-standing pneumonia. In a preliminary account of the transference of some of the British strains to mice (Glover and Andrewes, 1941), it was noted that they are not so readily adapted to this species as are the American swine strains and so in this respect they appear to resemble human influenza strains.

The swine strains studied by Shope have been compared with a standard human influenza A virus by other workers, using serum neutralization tests, testing of mice by intranasal instillation of 1–10 M.L.D. after intraperitoneal vaccination with serial doses of virus (Eaton and Pearson, 1940), and the red-cell agglutination technique of Hirst (1942). Although the human and swine strains possess a common antigenic component, it is agreed generally that these methods serve to distinguish them.

In the present paper the results will be given of neutralization and vaccination experiments in which the recently isolated British strains of pig virus are compared with human influenza A strains and with Shope's porcine virus, strain 15.

ESTABLISHMENT OF THE VIRUSES IN THE MOUSE

Several pig strains were received from Cambridge and N. Ireland, but one only from each locality was selected for detailed study. The Cambridge virus after ten passages through ferrets, in which it set up characteristic fever, nasal catarrh, and secondary pneumonia (Blakemore and Gledhill, 1941), produced lesions in mice after three passages. Ferrets were readily infected with the N. Ireland material, represented by the consolidated lungs of naturally or experimentally

infected pigs, and showed a typical diphasic febrile response. A lung and turbinate suspension from the first ferret failed to infect mice, but on further passage through ferrets the virus became more easily adapted to mice until eventually two strains were successfully established; it was not, however, until a number of passages had been made in these animals that frank pneumonic lesions were observed in them. The concentration* of virus in the lungs was determined on several occasions. In the early passages in mice the titre was fairly low, namely, between 10^{-2} and 10^{-3} (the titre was taken as the 50 per cent. mortality end-point as calculated by Horsfall, 1939); the infectivity of the lungs then increased until both the Cambridge (Cl)† and N. Ireland (N.I.) strains regularly titrated at 10^{-5} to 10^{-6}, reaching the level attained currently by the Shope virus. Later there was a general fall in infectivity which was most marked with Cl strain.

Ferrets, recovered from infection after intranasal instillation of the viruses and afterwards injected I.P. with one reinforcing dose of high titre mouse lung, provided the immune sera against the different strains. The ferrets were bled 14 days after injection, and the sera inactivated at 56° C. for 30 minutes, filtered, and preserved with merthiolate (1 : 10,000).

As the experiments so far completed have not indicated conclusively the relations of the above porcine strains to the Shope strain on the one hand, and to the human strains on the other, no more than a rough pattern of their antigenic structure can be suggested at the present stage of our work.

NEUTRALIZATION EXPERIMENTS

The two British porcine strains were compared with the Shope porcine strain in the first place. Ferret anti-serum, in fivefold dilutions, ranging from 1/2 to 1/1250, were mixed for test with the homologous virus and the heterologous viruses in 10^{-2} suspensions of infected lung; the titration end-points of the lung tissues were: Cl, 10^{-3}; N.I., 10^{-6}; Shope, 10^{-4}. Six mice were injected intranasally with each mixture. The potency of each serum was taken as corresponding with the highest dilution at which 50 per cent. of the mice were protected. The results showed that each serum exerted a stronger protective effect against its homologous strain of virus than against either of the heterologous strains. On the whole, the British strains seemed more closely related to each other than was either to the Shope strain. The results of these neutralization

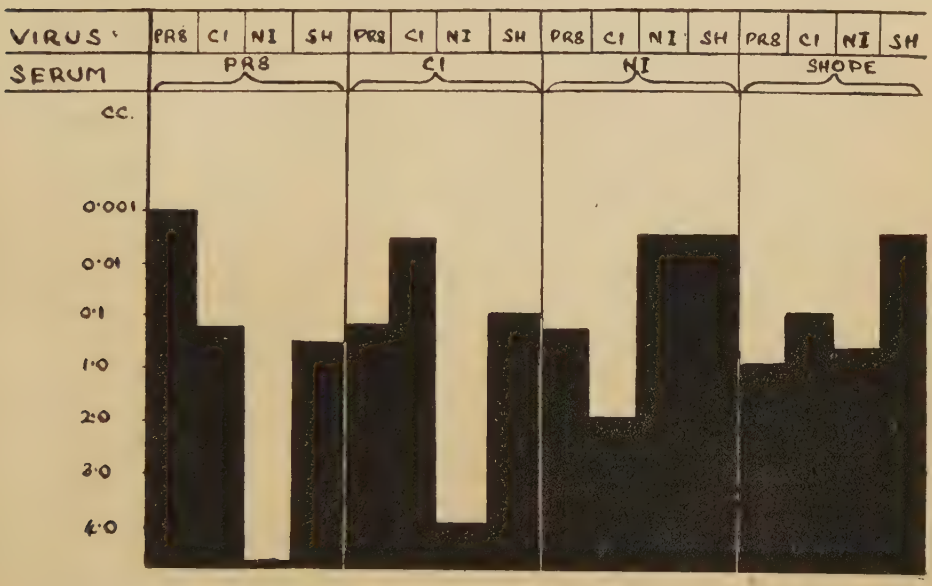
* In this paper all calculations on the virus content of mouse lungs refer to 5 or 10 fold dilutions of weighed amounts of lung in broth saline or 10 per cent. serum broth.

† The strain used was labelled Cl by Blakemore and Gledhill (1941) and was employed by us in our preliminary mouse work (Glover and Andrewes, 1941). It is designated Ca in a recent paper by Hudson, Sigel and Markham (1943).

tests may not have been, however, entirely trustworthy since slight differences in the antigenic composition of related sera may not be revealed if the amount of test virus which is administered is too great.

A second experiment was carried out, therefore, with the object of overcoming this difficulty. Each virus, at an estimated concentration of 1,000 M.L.D. (calculated on the 50 per cent. mortality basis) was mixed for test with fivefold dilutions of serum, and in this way the neutralizing capacity of each serum for its homologous strain of virus and for the heterologous strains was computed, the PR8 (human influenza A) strain of virus and its immune serum being also included in the series. In effect, the concentration of virus in some of the suspensions slightly exceeded 1,000 M.L.D. Accordingly, the results which are displayed graphically in Fig. 1, show

FIG. 1. Quantity of Serum necessary to neutralise 10,000 doses of Homologous or Heterologous Virus.



the calculated amount of serum required to neutralize 10,000 M.L.D. of virus.

Again, in this experiment it will be observed that each strain was most effectively neutralized by its homologous anti-serum, an exception being that the N.I. anti-serum was as potent against the Shope strain as it was against the N.I. strain itself. Otherwise, the heterologous strains were all neutralized by an anti-serum at a lower level of dilution than the homologous strain. It will be noted in particular that PR8 anti-serum was almost without any effect on N.I. strain.

Several standard ferret sera prepared against human influenza

strains, *viz.*, W.S., Tal, Gat., and Chr, and human convalescent sera, known to be potent against the Shope strain, neutralized the Cl strain at about the same titre as they did the Shope strain.

CROSS-VACCINATION EXPERIMENTS

(a) *Tests by Pulmonary Route*

As the above neutralization tests had failed to display clearly the relations of the several strains of virus, it was hoped that more accurate information upon their antigenic composition might be forthcoming from the results of vaccination experiments. The fairly close relationship between the Shope strain and the British strains revealed by the neutralization tests was not completely confirmed by a preliminary vaccination experiment. Mice immunised by two successive I.P. inoculations of the live Cl strain were completely protected against an I.N. instillation of a 1/100 lung suspension of the Cl strain, whereas the Shope strain even in 1/1000 suspension produced death or widespread pulmonary lesions in similarly immunized mice. Further experiments were therefore undertaken, as follows :—

Method.—Groups of 36 mice were immunized with two successive doses of infected mouse lung suspensions (0·2 c.c. subcutaneously, 0·5 c.c. intraperitoneally). About 1,000 M.L.D. for the PR8 strain and 100 for the Cl and Shope strains were estimated to have been inoculated. Fourteen days after the second dose the survivors were divided into groups and were tested, together with controls of the same weight, by the I.N. instillation of 0·05 c.c. of a 1/100 virulent lung suspension. The virus end-points were 10^{-6} for the PR8, 10^{-4} for the Cl, and 10^{-3} for the Shope infective lungs.

The results showed that mice vaccinated against Cl were almost completely immune (90 per cent. protected) against the same strain, but were only slightly protected against PR8 (8 per cent.) and against Shope (2 per cent.).

Mice vaccinated with PR8 were moderately immune against the homologous strain (73 per cent.) and against the Cl strain (82 per cent.), whilst against the Shope strain their susceptibility was appreciably higher (33 per cent.).

Mice vaccinated with the Shope strain were completely refractory to the homologous virus (100 per cent.), but they only partially resisted PR8 (27 per cent.) and were entirely susceptible towards the Cl strain (0 per cent.).

As the mode of testing was somewhat drastic, it was appreciated that different and more dependable results might have been obtained if the dose employed had been regulated so that quantitatively it did not greatly exceed the minimum required to set up infection. Several workers have affirmed that slight differences in the antigenic make-up of influenza strains can only be detected when the test doses employed are small. Eaton (1940) has used stock suspensions containing not more than 10 and not less than 1 M.L.D., preserved at

−76° C. in a solid CO_2 chest. As a low temperature chest was not available for our use, it was not easy to obtain virus suspensions that fell within the desired range. Moreover, unlike the American workers, we did not always obtain a sharp end-point in titrations upon control mice, and this added another biological complication.

The following tests illustrate the various difficulties encountered :—

Suspensions of mouse lung infected with each strain were injected I.P. (in one inoculation) into groups of 50 mice, in tenfold dilutions, representing estimated concentrations of 10, 100, 1,000, 10,000 M.L.D. (the tenfold dilution was sometimes omitted); each strain was titrated I.N. simultaneously. Fourteen days after I.P. vaccination, the survivors were subdivided into smaller groups, to be tested with the same suspensions, meanwhile stored in solid CO_2 in a thermos flask, and each then diluted to an estimated level of 1 to 10 M.L.D. Ten days later, all survivors in the control and vaccinated groups were killed, and the results, depicted in Table I, may be summarised as follows :—

TABLE I

VACCINATION OF MICE : INTRANASAL TEST DOSE

Vaccine	*Test Virus*	*No. of M.L.D. in Test Doses (50 per cent. Lethal End Point)**	*Effective Vaccinating Dose*	*Ratio Homologous to Heterologous Virus*
PR8	PR8	1/2	100	
	Cl	1/2	100-1,000	1 : 1 to 1 : 10
	N.I.	1/4	1,000	1 : 10
	SH	2/3	10,000	1 : 100
Cl	W.S.	1/3	1,000-10,000	1 : 10 to 1 : 100
	PR8	10	10,000	1 : 100
	Cl	1/2	100	
	N.I.	1/2	1,000	1 : 10
	SH	1/5	100-1,000	1 : 1 to 1 : 10
N.I.	W.S.	5	10,000	1 : 100
	PR8	5	10,000	1 : 100
	Cl	2	1,000-10,000	1 : 10 to 1 : 100
	N.I.	2	100	
	SH	1	100-1,000	1 : 1 to 1 : 10
Shope	PR8	2	10,000	1 : 100
	Cl	1/2	100-1,000	1 : 1 to 1 : 10
	N.I.	1	1,000-10,000	1 : 10 to 1 : 100
	SH	1/3	100	

* Calculated by Reed and Muench method.

Group I (vaccinated with PR8).—The controls reacted satisfactorily, excepting those of the N.I. group, in which the mortality was low. Nearly complete protection was given against the homologous virus. The degree of immunity afforded against the Shope strain was low, while against Cl and N.I. it was intermediate. No test was made against W.S.

Group II (vaccinated with Cl).—There was nearly complete protection against the homologous strain. The doses of the PR8 and Shope strains selected for testing immunity were not quite satisfactory, because well over 50 per cent. of the controls subjected to the test dose (a 10^{-5} dilution) of the former strain and less than 50 per cent. of those receiving the latter strain, died. The vaccinated mice tested with PR8 were poorly protected, the immunity being less than in those tested with W.S., but this difference may have been caused by the more severe test dose employed of the former strain. Against tests with the Shope strain there appeared to be some protection, and a still better immunity against the N.I. strain, but a difference in the severity of the test doses may account for the apparently stronger protection.

Group III (vaccinated with N.I.).—On the whole, the control mice reacted satisfactorily. Vaccination afforded complete protection against the homologous strain of virus, an appreciable protection against the Cl and Shope strains, and a lower degree of protection against the PR8 and W.S. strains. (The mice were, however, subjected to somewhat more severe test doses of virus.)

Group IV (vaccinated with Shope).—The death-rate in the control mice infected with the test dose of the Shope strain was rather low for satisfactory comparison. Nevertheless, some immunity was conferred against the Cl strain, but little protection against the PR8 and N.I. strains.

(b) *Tests of Immunity by Intraperitoneal Inoculation of Virus*

Rickard and Francis (1938) showed that the PR8 strain of virus could be recovered from the lungs in high concentration, and pulmonary lesions, not resulting in death, were not uncommon in mice that had been inoculated intraperitoneally with 50,000 to 1,000,000 intranasal M.L.D. The virus reached its highest concentration in the lungs 48 to 72 hours after inoculation and sometimes persisted until the tenth day. This knowledge suggested a further method of ascertaining the antigenic structure of the various strains. It seemed possible that while the protection evoked by an initial inoculation of a small dose of a given virus might ward off pulmonary invasion following subsequent inoculation of a large dose of the same strain, it might not be sufficient to stem infection by a heterologous strain.

Methods.—Groups of mice were vaccinated intraperitoneally with graded doses of live virus belonging to the various strains used above, and afterwards inoculated by the same route 10 to 14 days later, the titre of virus present in the lungs after two or three days being then ascertained in the usual way, by I.N. instillation of tenfold dilutions of the pooled lungs of each experimental batch.

Quantity of Virus Present in the Lungs after an I.P. Inoculation

Before the main vaccination experiment was undertaken it had to be determined what was the relationship between the amount of

virus injected I.P. and invasion of the pulmonary tissue. Mice, in groups of 20, were inoculated I.P. with 50,000, 250,000 and 1,000,000. I.N. mouse M.L.D. of the lungs of mice injected with PR8. Five mice from each group were killed at 24-hour intervals and the pooled lungs from the separate groups suspended in 10 per cent. broth saline. Tenfold dilutions of the supernatant fluids, after centrifugation, were then instilled intranasally into mice, five being tested with each fluid. The survivors were killed on the tenth day.

It was found that after 24 hours, no virus was recovered from the lungs of the mice that had received 250,000 M.L.D. but in those injected with 1,000,000 doses, virus was detected in dilutions up to 10^{-5}. After 48 and 72 hours, extensive pulmonary invasion (10^{-6}) was found to have followed I.P. inoculation of the smallest dose employed. Thenceforward, the virus rapidly disappeared from the lung, hardly any being recoverable on the seventh day. In spite of the large amount of virus present in the lungs, there was a complete absence of visible lesions at the 24th and 48th hours. They began to appear in the lungs at the third and fourth days when their extent varied from pin-point haemorrhages to consolidation of one-third to one-half of the lung tissue in individual mice. The maximum dose of virus which had to be injected I.P. into normal mice to ensure pulmonary invasion was also ascertained. Mice, in groups, were killed on the third day after I.P. inoculation of graded doses of virus and the concentration present in the lungs determined.

The results suggested that there was a firm obstacle to the localization of virus in the lungs, but that when once this barrier was overcome multiplication of virus in the lungs proceeded as after I.N. instillation. Likewise, a distinct tendency towards an " all or nothing " effect was suggested : in other words, if virus succeeded in gaining access to the lungs it was likely to be present in them in high concentration but if absent in high dilutions it was not likely to be detected in concentrated suspensions. Very similar results were obtained with the strains other than PR8 tested in this way, and from them it was deduced that a dose not less than 50,000 M.L.D. must be inoculated I.P. to ensure transport of sufficient virus into the lungs for its detection at a dilution of 10^{-2} and above.

Vaccination Experiments

The vaccination experiments were undertaken to determine the strength of the immunity induced by an initial I.P. inoculation of virus to a subsequent inoculation of the same virus or of a different strain by the same route.

Mice in groups of five were injected I.P. with suspensions of PR8 mouse lung in graded concentrations of 10^{5} to 10^{1} intranasal M.L.D. Fourteen days later they were again inoculated I.P. with about 250,000 M.L.D. of the same strain, together with controls of a similar weight. On the third day after this injection all were killed, and pooled suspensions of the lungs of each group were

titrated. Complete protection was afforded by 10^5 and 10^4 doses I.P., partial protection by 10^3 doses, while the 10^2 and 10^1 doses were completely ineffective. The sharp line of demarcation between the 10^2 and 10^4 level was striking.

In order to test the strength of the immunity set up, in a similar way, against a second I.P. inoculation with heterologous strains, mice were next given, in groups, a first or vaccinating injection, of graded doses of each strain. Each group was then divided into batches to be tested severally with the homologous and the heterologous strains administered as a 10 per cent. suspension of infected lung. No uniformity in the total intranasal M.L.D.s given in this way could, however, be guaranteed because the different strains varied in their infectivity for mice. The Cl and Shope strains invariably gave a lower titration end-point although both were well established in mice.

The results (see Table II) show that :—

TABLE II

VACCINATION OF MICE : I.P. VACCINATION FOLLOWED BY I.P. TEST DOSE

Vaccine	*Test Virus*		*Effective Vaccinating Dose*	*Ratio : Homologous to Heterologous Virus*
	Strain	*Amount injected, M.L.D.*		
PR8	PR8	250,000	1,000	
	Cl	50,000	100,000	1 : 100
	N.I.	250,000	>100,000	1 : 10 to 1 : 100
	SH	150,000	>100,000	1 : 10 to 1 : 100
N.I.	PR8	200,000	100,000	1 : 100
	Cl	50,000	1,000	1 : 1
	N.I.	200,000	1,000	
	SH	100,000	10,000	1 : 10
SH	PR8	250,000	50,000	1 : 100
	Cl	75,000	50,000	1 : 100
	N.I.	200,000	50,000	1 : 100
	SH	100,000	5,000	

(*a*) PR8 used as vaccine protected moderately against itself, and very slightly against Cl but did not protect against N.I.

(*b*) N.I. used as vaccine protected equally well against itself and against Cl. It gave some protection against Shope but almost none against PR8.

(*c*) Shope used as vaccine was effective against itself and almost ineffective against the other three strains of virus.

CHICK RED-CELL AGGLUTINATION TESTS

During the course of these experiments, Miss D. Lush kindly tested these viruses and their corresponding antisera by the chick

red-cell agglutination test of Hirst (1942). Details of the method of applying this test to the analysis of the relationship of different strains of human influenza and Shope viruses respectively have already been given by several workers.

The viruses were propagated in developing hen's eggs which were inoculated allantoically with 0·1 c.c. of a suitable dilution of infective material. The fluid was harvested with the usual precautions to avoid haemorrhage. The assessment of the agglutinating power of the virus suspensions and the degree of serum inhibition by homologous and heterologous sera closely followed the scheme adopted by Hirst. Table III shows the results of these tests. With the exception of the N.I. virus which was inhibited by Shope antiserum, each strain was much more effectively neutralized by the homologous than by the heterologous sera.

TABLE III

SERUM INHIBITION (HIRST METHOD)

Serum	*PR8*	*C1*	*N.I.*	*SH*	*Virus*
PR8	100	12·5	25	12·5	
C1	<3·12	100	12·5	12·5	
N.I.	<1·0	4·68	100	18·75	
SH	9·37	50	100	100	

Figures expressed as values per cent. of homologous virus.

DISCUSSION

The earlier studies on influenza virus (Francis and Shope, 1936; Smith, Andrewes and Laidlaw, 1935; Rosenbusch and Shope, 1939), showed that human influenza A virus could be readily differentiated from swine influenza virus by serum neutralization and vaccination tests in mice. Eaton and Pearson (1940) in confirming and extending later findings of Francis (1939) point out that experiments on active immunization of mice by the I.P. route indicate that the relationship between certain human strains is determined by common antigens which are present in the proportion of one part in the heterologous to ten parts in the homologous strain. On the same basis, the amount of human strain antigen in swine influenza virus is approximately 1/1,000th of the quantity in human antigen; conversely the latter contain 1/10 to 1/100 as much swine antigen as the Shope strain.

The results of the experiments outlined in this paper seem to show that strains isolated from pigs in the British Isles have a complex antigenic structure and, generally speaking, fall between the authentic human strains of recognized type, *e.g.*, PR8, W.S., etc., and the Shope porcine strain. The transference of these strains to ferrets can be effected fairly easily but their establishment in mice requires several serial passages before overt pneumonic lesions are produced. In

this respect they seem to bear a closer resemblance to human than to the American swine viruses. Nevertheless, the various methods of assessing the exact relationship of this group to established strains have, so far, failed to give a clear picture since each method employed has given a slightly different answer. Various explanations of the divergent results might be advanced. Irwin and Golden (1942) have offered evidence that different individuals of a species may respond very differently to minor components of a complex antigen. If this is so, it is not surprising that immunological studies of complex antigens have often given conflicting results. In the case of influenza viruses, the irregularity of neutralization and other tests may be due to variations in the ability of individual ferrets and mice to respond to different minor antigens in the virus structure. In addition, the work of Francis (1939), Eaton and Pearson (1940) and others indicates that unless the relative proportions of virus and serum in neutralization tests or the number of infective doses used to measure the resistance induced by vaccination, are carefully graded, there is little possibility of detecting slight differences between closely related strains.

The results of the present investigation have been summarized in Table IV, in which the various methods described in this paper are roughly compared. The final columns show the results obtained by Hudson, Sigel and Markham (1943) in which the same strains were examined by the Hirst technique using ferret antisera prepared in this country and fowl antisera prepared in the U.S.A. It seems clear that two groups of workers using the same method may obtain rather different answers. Some of the factors which may be responsible for such discrepancies in the red-cell agglutination test have been evaluated recently by Stuart-Harris (1943).

However, results obtained by all methods contrast with those of Shope (1939), who found that from the antigenic point of view the American porcine strains formed a very homogeneous group, when studied by means of convalescent (swine) sera.

Interesting questions arise concerning the relationships between human and porcine viruses. Laidlaw (1935) and Shope (1936) have discussed the possibility that the American (Shope) porcine virus may be a direct descendant of a human 1918 pandemic influenza virus. Shope has shown that pigs are susceptible to infection with human viruses both by direct inoculation experiments (Shope and Francis, 1936), and indirectly by finding antibodies to human A viruses in pigs in contact with a human outbreak, Shope (1938). Our finding of fairly close relations between human A and British porcine viruses raises a question as to whether at this time an exchange of viruses between the two hosts may not be taking place. We have, however, no epidemiological evidence to support this idea; outbreaks of swine influenza have been occurring every year in the British Isles, while influenza A was not recognized over here in 1940 or in 1942.

TABLE IV

SUMMARY OF EXPERIMENTS

Serum or Vaccine	*Test Virus*	*Neutralization*	*Vaccination*		*Hirst Method*		
			Test Dose I.N.	*Test Dose I.P.*	*Ferret Serum†*	*Ferret Serum**	*Fowl Serum**
PR8	W.S.	Not done	Not done	Not done	Not done	++	±
	PR8	++++	++++	+++	++++	++++	++++
	Cl	+++	+++	+	±	++	+
	N.I.	O	++++	O	+	+++	+
	SH	+++	+	O	±	O	—
Cl	W.S.	Not done	+	Not done	Not done	±	++
	PR8	+++	±	Not done	O	±	+++
	Cl	++++	+++	Not done	++++	++++	++++
	N.I.	+++	+	Not done	±	+	++
	SH	+++	+++	Not done	±	?	? ±
N.I.	W.S.	Not done	+	Not done	Not done	+	++
	PR8	+++	++	O	O	+	++
	Cl	++	++	+++	±	+	+
	N.I.	++++	++++	+++	++++	++++	++++
	SH	++++	+++	+	+	±	±
Shope	W.S.	Not done	Not done	Not done	Not done	++	±
	PR8	++	+	O	±	+	+
	Cl	+++	+++	O	++	+++	O
	N.I.	++	+	O	++++	++++	±
	SH	++++	+++	+++	++++	++++	++++

I.N. = Intranasal.
I.P. = Intraperitonea'.

† Tests by Lush, D.
* Tests by Hudson, Siegel and Markham.

Protection

++++ = 100 per cent.
+++ = 75 ,,
++ = 50 ,,
+ = 25 ,,
± = Less than 25 per cent
O = None

SUMMARY

Viruses recovered from outbreaks of influenza in pigs in the British Isles have been established in the ferret and in the mouse. They produce a febrile catarrh in the ferret and pulmonary consolidation in mice resembling the changes induced by influenza A virus or the porcine virus of Shope.

These viruses have been compared with standard strains (W.S., PR8, and Shope) by neutralization and vaccination tests in mice and the inhibition of fowl red-cell agglutination (Hirst technique).

The neutralization and red-cell tests indicated that in general each strain was most effectively neutralized by its homologous antiserum. Vaccination experiments in which mice were tested for immunity either by the intranasal or by the intraperitoneal route, gave results which suggested that homologous vaccines were 10 to 100 times as effective as heterologous ones.

It is still difficult to obtain a clear conception of the antigenic relations of these viruses. The impression has been gained that neither of the two strains examined is more closely related to the other or to the Shope pig virus than it is to a human influenza A (PR8) virus.

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